THE UNIVERSITY OF ALBERTA

RADIOSENSITIZATION EFFECTS OF HALO-ANALOGUES

ON CALF THYROID CELLS <u>IN</u> <u>VITRO</u>

by

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The undersigned certify that they have read,
and recommend to the Faculty of Graduate Studies for
acceptance, a thesis entitled "Radiosensitization effects
of halo analogues on calf thyroid cells _in vitro_,"
submitted by Russell Emil Giblak, in partial fulfillment
of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The present investigation shows that within
certain limits, the persistent chromosome bridge forma-
tion frequency (CB) can be used as a parameter of
x-irradiation damage to calf thyroid cells in an
in vitro system. Based on this parameter, damage in
this system was found to reach a maximum at 250R.

On the basis of survival studies deoxyribose
nucleic acid (DNA) base analogues, such as 5-iodo-2'-
deoxyuridine (IUDR) and 5-bromo-2'-deoxyuridine (BUDR),
are known to enhance x-irradiation damage to other
in vitro mammalian cell cultures. The present investi-
gation ascertains whether or not this enhancement
effect is detectable on a cytological basis. As a
prerequisite of such determination a growth study was
carried out with IUDR pre-treated thyroid cells.
Growth was found to be normal at a 4 µg/ml concentration
of IUDR, a concentration which subsequently proved
to enhance x-irradiation damage to the thyroid cell
system.

Although the cytological response of calf
kidney cells in vitro to x-irradiation is similar to the
one obtained with thyroid cells, the toxicity of IUDR
to kidney cells appears to be higher than that found
for thyroid cells. However, upon treatment of kidney cells
with IUDR, and subsequent x-irradiation, the kidney cells
show a seemingly lesser degree of radio-sensitization than
was found for thyroid cells.

Since 5-iodo-2'-deoxycytidine (ICDR) is believed
to have certain in vivo advantages, its effects on thyroid
cell growth and radio-sensitisation were of interest.
ICDR appears to be less toxic to thyroid cell growth than
IUDR. However, the degree of x-irradiation enhancement

resembles that found for IUDR. A combination of IUDR
and ICDR was not found to be any more efficient in radio-
sensitization of thyroid cells than the use of either
analogue alone. Therefore, no conceivable advantage
could be achieved by the use of such a combination.

Damage caused by continuous 131I-irradiation
resembles the damage found after x-irradiation. However,
IUDR pretreatment appears to be rather inefficient in
enhancing damage by 131I-irradiation. It is proposed
that the difference in radiation enhancement between
x-irradiation and continuous 131I-irradiation is due
to the difference in interaction between IUDR-containing
DNA and the two forms of irradiation.

From clone-forming experiments with thyroid
cells, it appeared that x-irradiation damage correlates
well (under the various conditions evaluated) with the
cytological observations and suggests the existence of
a relationship between CB and reproductive death.
These experiments also show that damage due to 131I-
irradiation does not appear to be potentiated by IUDR.
This latter finding is in agreement with cytological find-
ings. However, the amount of reproductive death after
131I-irradiation (with and without IUDR pre-treatment) is
higher than would be predicted on the basis of cytological
evidence. This disparity may be explained as a reflection
of cell attachment inhibition.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

TABLE OF CONTENTS cont'd

Page

LIST OF TABLES, FIGURES AND PLATES cont'd

LIST OF TABLES, FIGURES AND PLATES cont'd

LIST OF TABLES, FIGURES AND PLATES cont'd

LIST OF TABLES, FIGURES AND PLATES cont'd

INTRODUCTION

Soon after the discovery of x-rays, it was found that radiation had a profoundly damaging effect on mammalian tissue. The discovery that neo-plastic tissue was damaged to a much greater extent than normal tissue following irradiation led to the formulation of a law stating that: "X-rays are more effective on cells which have a greater reproductive activity, the effectiveness is greater on those cells which have a longer dividing future ahead, and those cells, the morphology and function of which are least fixed" (Bergonie Tribondeau, 1906). Although this law has now been modified, its basic premise is still valid.

Muller and Stadler (1927,1928) independently discovered that x-rays induce mutations and chromosomal aberrations among which the poly-centric chromosome resulting from abnormal chromosome restitution. In addition, with the advent of the ingeneous tissue culture techniques of Puck and Marcus (1955, 1956), Marcus, Cieciura and Puck (1956) and Puck, Marcus and Cieciura (1956), the radiosensitivity of mammalian cells (i.e., in terms of cell survival) has been more accurately evaluated. These studies have shown that cell survival is related to x-irradiation dose. Furthermore, with improved cytological techniques, Puck (1960) showed that relatively low doses of x-rays can cause chromosomal breakage. These breaks, and subsequent abnormal restitution, can result in the formation of di- and poly-centric chromosomes (CB).

Koller (1947) and Whitfield, Rixon and Rhynas (1959) showed that persistent chromosome bridge formation was a cytological consequence of radiation damage in cultures of L strain mouse fibroblasts. They also

indicated that this parameter of x-irradiation damage
could be obscured by doses exceeding a certain maximum
level, and suggested that one reason for this might be
extensive damage to the mitotic spindle. The immediate
consequence of mitotic spindle damage would be the
failure of post irradiation mitosis, which would render
impossible the cytological detection of chromosome
bridges.

Atwood (1963) reported that an x-ray dose of
300R resulted in a high proportion of di-centric and
poly-centric chromosomes in Tradescantia. Yu (1966)
also showed a similar x-ray induced consequence in
Chinese hamster cells cultured in vitro.

The early work of Greer and Zamenhof (1957)
and Greer (1960) has shown that 5-bromouracil (BU) can
be introduced into the newly synthesized DNA of E. coli,
and that this fraudulent DNA had the effect of increasing
the sensitivity of the organism to ultra-violet light.
Following this investigation, Opara-Kubinska, Lorkiewicz,
and Szybalski (1961) demonstrated that incorporation of
5-halodeoxyuridines enhanced the radiosensitivity of
transforming DNA. Further instances of this phenomenon
were found in bacteriophages, (Litman and Pardee, 1960)
and in bacteria, (Smith and Tomlin, 1962). These find-
ings stimulated parallel investigations on the incorpora-
tion of 5-haloanalogues into mammalian cell DNA. Eidinoff,
Cheong and Rich (1959) have shown 5-iodo-2'-deoxyuridine
(IUDR) and 5-bromo-2'-deoxyuridine (BUDR) incorporation
into the DNA of a strain of human cells (H. Ep #1) derived
from cervical carcinoma. A similar incorporation of BUDR
into Hela and KD cells has been shown by Littlefield and
Gould (1960). They also have indicated that increased
concentrations of the analogue resulted in an increased
toxicity. Growth inhibition (based on total protein) of
mammalian cells by the introduction of BUDR and IUDR, was

shown by Cheong, Rich and Eidinoff (1960). From the
investigations of Djordjevic and Szybalski (1960) and
Erikson and Szybalski (1961) it has been found that
mammalian DNA, which contained BUDR and IUDR, was more
sensitive ionizing as well as non-ionizing (U.V.)
irradiation (based on cell survival). These authors also
concluded that BUDR is superior to IUDR as a potentiator
of ultra-violet light damage, and that the converse is
true for x-rays. They pointed out, however, that IUDR
was more toxic than BUDR to mammalian cells, thus
nullifying radiosensitizing effect.

Pre-treatment with 5-halodeoxyuridine has
been shown to enhance chromosomal aberration in Vicia
faba by Kihlman (1963), in Zebrina pandula by Koo (1963),
in barley by Kaul and Natarajan (1966), and in mammalian
cells by Ragni and Szybalski (1962), Dewey and Humphrey
(1965), and Chu (1965a).

Following the synthesis of halogenated deoxy-
cytidines (Frisch and Visser [1959] and Chang and Welch
[1961]), investigations were carried out (Cramer, Prusoff
and Welch [1961], and Cramer, et al., [1962]) which showed
that these bromo- and iodo analogues of deoxycytidines
were incorporated into the DNA of mammalian cells after
conversion to BUDR and IUDR respectively. Growth deter-
minations (Cramer, et al. 1962) indicated that these
analogues were less toxic than their deoxyuridine counter-
parts. At the same time, Kriss, Tung and Bond (1962)
found that 5-iodo-2'-deoxycytidine (ICDR) was degraded less
extensively in the mouse (in vivo) than IUDR, and the
amount of both which were incorporated into DNA was
similar. From the elegant studies on D98/AG cells carried
out by Erikson and Szybalski (1963), it became clear that
the amounts of incorporation of deoxycytidine and deoxy-
uridine analogues were similar. Moreover, these authors

concluded that the radiosensitizing potential was much
the same.

Moore and Colvin (1966) exposed Chines ham-
sters to 131I-irradiation (in vivo) for various periods,
after which the thyroids were removed, cultured, and
evaluated for irradiation damage (in vivo). Their
assessment indicated that 131I-irradiation induced the
formation of chromosomal aberrations particularly of
di-centric chromosomes among other types. A further con-
clusion drawn by these authors was that the thyroid was
sensitive to low doses of this 131I-irradiation.

It must be clarified at the outset that the
prime purpose of the present in vitro study was to eval-
uate iodo-analogues as radiosensitizers which, in the
future, might be of some value in thyroid tumor therapy.
Other reasons for choosing the thyroid as the organ of
study are as follows:

    a. It was felt that the thyroid cells might
       be refractory to IUDR cytoplasmic toxicity
       (see sub. c).

    b. Mitotic turnover of normal thyroid cells
       is rather slow when compared to the turn-
       over of tumor cells (Kasza, 1964). Since
       IUDR and ICDR are incorporated only by
       newly-replicating DNA, the increased turn-
       over rate of tumor cells would result in
       an obvious advantage in any therapeutic
       application.

    c. It has been reported by Hung and Winship
       (1964) and Assem (1965) that thyroid cells
       in vitro maintain their ability to concen-
       trate iodide, and to synthesize iodotyro-
       sines and iodothyronines. Tong, Kerkoff
       and Chaikoff (1962) and later, Mallette

and Anthony (1966) have shown that thyroid
cells maintain their ability to reaggregate
and reconstitute into functional follicular-
like formations.

To assess damage from ionizing radiation, both
growth and cytological studies were carried out, using
the frequency of chromosomal bridge formation (CB) as a
parameter. The results of these investigations led to a
comparison between the cellular damage caused by x-irrad-
iation alone and the cellular damage resulting from
x-irradiation and IUDR pre-treatment. Since the parameter
of irradiation damage was based on cytological evaluation
(which in turn was dependent on the mitosis of thyroid
cells), information concerning the effect of IUDR on
growth was necessary. In order to compare the responses
of two autologous cell systems, a further comparison of
the IUDR effect on growth and radiosensitivity was made
between calf thyroid and kidney cells.

Because ICDR is reportedly less toxic, less
extensively degraded and just as effective in maximizing
irradiation damage, a comparison between the effects on
growth and radiosensitivity of ICDR and IUDR was made.

The effect of a combination of IUDR and ICDR
on thyroid cell growth and radiosensitivity also was
explored. The results were compared with those obtained
concerning the effects of x-irradiation alone, and
x-irradiation following pre-treatment with either of the
aforementioned analogues. It was thought that this might
yield independent information regarding the mode of
incorporation of the two moieties (ICDR and IUDR).

A comparison between the abilities of 131I and
x-irradiation to induce chromosomal damage in IUDR
pre-treated and untreated cells was carried out. It was

hoped that this assessment might yield information about the relative efficiency and mode of action of the two forms of radiation.

Finally, a clone-forming study on thyroid cells was carried out in order to provide some indication of the relationship between the cytological observations and that of reproductive capacity.

MATERIALS AND METHODS

I. Material: Source of Tissue

Thyroid samples were taken from calves of both
sexes randomly. All calves used in these experiments were
from 4 to 8 months old (post partum) without regard to
breed.

II. Preparation of Tissue Samples

A. Collection

Thyroid glands or lobes of thyroid glands obtained
from local slaughter houses were excised with sterile
scissors. The thyroid lobes (weighing between 10 and 30
grams) were immediately rinsed in cold tap water. This
latter procedure prevented gross contamination of the cul-
tures which plagued many of the early experiments in this
work. After rinsing, the lobes were placed in cold
Dulbeccos Balanced Salt Solution (BSS, 1st change) con-
taining phenol red as a pH indicator and 100 units each of
penicillin and streptomycin. Mg and Ca ions were omitted
from the BSS (Merchant et al, 1960).

B. Processing

Immediate processing of the thyroid samples was
imperative as it was found that a delay of more than 3 to
4 hours decreased cell viability. The culture technique
adopted for this study was similar to that for human
thyroid cells, with some modifications described by
Pulvertaft et al., (1959), and Irvine, (1960a). All
samples were processed in a room sterilized by ultra-
violet light. Once the samples were obtained, seven ster-
ile petri dishes were set out. Four contained approximately
20 ml of BSS. Two were empty and one contained 40-50 ml
of absolute ethanol. The thyroid samples were thoroughly

washed in the 1st change of BSS and then transferred by
means of sterile forceps to an empty petri dish. The
samples were then placed in absolute ethanol for a period
of 30 seconds, flamed and put into the second empty petri
dish. At this time all of the connective and fatty
tissue was removed together with the embedded parathyroid
glands. Subsequently, samples were transferred to a flask
containing the second change of BSS and washed thoroughly.
The necessity for washing, flaming and re-washing arose
because of the heterogeneous contamination problem orig-
inating at the slaughter house. The washed thyroid glands
were then transferred into the first of the four petri
dishes containing BSS (3rd, 4th, 5th and 6th change).
Progressive mincing by means of scissors was achieved with
each succeeding transfer. The fragments were then placed
in a 300 ml Erlenmeyer flask containing a teflon-coated
magnetic stirrer and 100 ml of 0.25% trypsin solution
(Baltimore Biological Lab., Baltimore, Maryland) in BSS.
Trypsinization (cell dispersal) was allowed to proceed
for 45 minutes at room temperature while being stirred on
a magnetic stirrer. This initial cell suspension was
discarded and the remaining thyroid gland fragments resus-
pended in 100 ml of fresh BSS supplemented with 0.25%
trypsin for further trypsinization. Magnetic stirring
was continued until the required number of cells in sus-
pension was obtained for each experiment. The trypsin-
BSS solution was replaced again after 90 and 105-120
minutes of stirring. The cell suspensions were strained
through gauze lined funnels and collected in 15 ml centri-
fuge tubes. The tubes were sealed with parafilm and
centrifuged at approximately 800 rpm for 15 minutes. An
International Clinical Centrifuge was used throughout.
After centrifugation, the supernatants were discarded
and the pellets of cells resuspended in Medium "199"

(Baltimore Biological Lab., Baltimore, Maryland) with the aid of gentle pipetting (Medium "199" with $NaHCO_3$ at pH 7.2 included an Earle's base, to which was added 100 units/ml of a penicillin and streptomycin mixture and 10% by volume of heat-inactivated calf serum). Various concentrations of 5-iodo-2'-deoxyuridine (IUDR) or 5-iodo-2'-deoxycytidine (ICDR) or both were added to the basic medium, depending on the requirements of any given experiment.

C. Staining and counting

After centrifugation and resuspension, a small sample of the cell suspension was withdrawn and cells stained with a citric acid-crystal violet stain (Merchant et al., 1960). Cell counts were made using a Spencer Bright-Line hemacytometer (American Optical Co.). As a general rule a minimum of three counts were averaged to determine the cell concentration. The cell number was then adjusted to between 8×10^4 and 10×10^4 cells/ml by the addition of Medium "199".

D. Cell-growth determination

A similar procedure to that outlined above was followed for growth determination experiments, except that in order to facilitate cell detachment, 0.5 ml of 0.25% trypsin solution was added to cultures at appropriate intervals following the initial five days of incubation (exceptions to this period are individually noted).

E. Dispensing and incubating cultures

Two ml aliquots were dispensed by pipette into (12 X 55 mm) Leighton tubes containing precleaned (10 X 50 mm) cover slips. The tubes were closed with silicone stoppers and incubated in a Precision Scientific, Model 805 Incubator at 37° for approximately five days before the medium was changed. During the five day incubation

period, the cells attached to the cover slips and grew.

For reasons of comparison, calf kideny cells were employed. Kidney cells were taken from calves of both sexes randomly. All calves used in these experiments were from 4 to 8 months old (post partum) without regard to breed. An identical technique as described above for calf thyroid cells was used for culturing of kidney cells. Hence, for in vitro studies primary cultures were employed.

III. Irradiation Procedures

A. Acute exposures to x-rays

After an incubation period of approximately five days, cultures were irradiated (prior to the customary change of medium at this time) using x-rays produced by a Phillips 300 KV constant potential x-ray unit, operated at 300 KV and 10 ma. The cultures were irradiated at a dose rate of 100 R/minute at a distance of 50 cm from the target. The doses used were: 125R, 250R, 500R and 1000R. The medium was changed following irradiation (0 hour) to minimize "secondary effects" of irradiation as they arise in an aqueous medium. At regular 12 hour intervals following irradiation, cover slips were removed from the tubes and stained.

B. Chronic exposure to 131I-irradiation

The procedure for 131I-irradiation was similar to that outlined for x-irradiation with some modifications. The 131I (carrier-free NaI) was added to the cultures at the time the medium was changed. The quantity added was approximately 9.85 μc/ml, which yielded a total accumulated dose of 301.2 rad after four days. The calculations were based on the following formula: (Hine and Brownell, 1956, p. 826)

$$D(t) = 73.8E_\beta C_o T_{eff} (1 - e^{-\frac{0.693}{T_{eff}}}) \text{ rad}$$

$D(t)$ - total absorbed dose of rays received by cultures in t (days)

$\overline{E}_\beta$ - average β particle energy in Mev

C_o - average initial concentration in $\mu c/cc$

T_{eff} (days) - physical half-life

The T_{eff} for 131I is 8.08 days. The cover slips were removed and stained as outlined below. Due to the relatively short half-life of 131I (8.08 days), the accumulated dose after 24 hours of incubation is high compared with the dose accumulated over the second, third and fourth 24 hours of incubation (as in x-ray studies).

Although theoretically the calculated dose from 131I is affected by the location of 131I (cellular or exogeneous) the average β-particle energy in Mev of 131I (0.178 Mev) is sufficiently high to offset any dose differential to 131I location in the Leighton tube (β max = 0.608 Mev [87.2%]; range in water [density = 1] approximately 2 mm).

IV. Hematoxylin-Eosin Staining and Mounting

Harris' Hematoxylin-Eosin staining procedure (Merchant et al., 1960) was employed. The cover slips were mounted on slides with Canada Balsam or "Euparal".

V. Cytological Evaluation

Cytological evaluation of the slides was carried out with a Leitz Orthoplan microscope equipped with phase contrast optics (400X). Radiation damage was assessed by scoring the percentage of chromosome bridges (CB). This was based on a count of at least 500 cells. The criteria for the evaluation of radiation damage originally included

the counting of pyknotic, binucleate and giant cells.
However, the latter parameters were found to be less re-
producible than persistent chromosome bridges.

VI.   Cloning Studies

        Since clone forming experiments yield informa-
tion on reproductive capacity, a technqiue was developed
to determine the ability of the cells to clone. Since
the conventional replating techniques, which are used for
established lines of cell cultures did not lend themselves
to primary diploid mammalian cell systems, modifications
had to be introduced. These modifications were designed
to avoid trypsinization of monolayers after the initial
five day incubation period, since primary diploid cell
cultures when trypsinized lose their potential for re-
attachment. Roller cultures were set up to keep the
cells in suspension. Ten ml aliquots of primary cell
suspension were dispensed into ordinary (15 cm X 15 cm)
pyrex dilution tubes and sealed by means of silicone
stoppers. The roller drum apparatus (New Brunswick Scien-
tific) was set at a speed of 50 rpm.(Parker, R.C.,1961).

        After five days of incubation, the rolled sam-
ples were centrifuged at 800 rpm for 15 minutes, and the
supernatant discarded. The cell pellets which had been
treated according to the following schedule were resus-
pended and 2 ml aliquots per Leighton tube were dispensed.

        1.   Cells cultured in Medium "199" (control)
        2.   Cells cultured in Medium "199" and sub-
             sequently x-irradiated
        3.   As sub. 1 but 4 µg/ml IUDR added
        4.   As sub. 2 but 4 µg/ml IUDR added
        5.   As sub. 1 but 4 µg/ml ICDR added

6. As sub. 2 but 4 µg/ml ICDR added

7. As sub. 2 but subsequently irradiated by addition of 131I (9.85 µc/ml) to the medium

8. As sub. 4 but subsequently irradiated by addition of 131I (9.85 µc/ml) to the medium

Thereafter, tubes were removed from the incubator at 24 hr. intervals. Non-adherent cells were removed by shaking action after which the medium was discarded and 0.5 ml citric acid-crystal voilet stain was pipetted into each Leighton tube. Staining was allowed to proceed for several minutes after which time the cover slips were inverted. The number of clones (i.e., as aggregate of two cells or more) in ten predetermined microscopic fields was counted (without removal of the inverted cover slips from the Leighton tube) using a magnification of 130 X. In order to avoid a biased observation the location of the fields to be examined microscopically, were indicated on the outside of the Leighton tubes.

VII. DNA Extraction

DNA extraction procedures used were essentially those described by Djordjevic and Szybalski (1960). The hot trichloroacetic acid (TCA) precipitation was carried out with 0.6M TCA and was repeated three times.

VIII. Chromatography

Calf thyroid cells were cultured for five days in the presence of a mixture of 4 µg/ml IUDR and 4 µg/ml ICDR, after which the cells were harvested. The procedure of hydrolysis by 6N HCl and subsequent paper chromatography was identical to the one described by Djordjevic and Szybalski (1960). Hydrolysates were spotted on number 1 Whatman paper by means of a micro-adaptor. Two dimensional ascending paper chromatography was used to separate the

hydrolysates from the DNA extraction. The chromatograms
were run for 5 hours in each direction after which they
were dried. A reflection photograph of the chromatograms
was made utilizing ultra-violet light of 2537 mμ wave-
length. Appropriate film (Panatomic X, Kodak) and devel-
oping (Dektol, Kodak) procedures were used.

IX. Liquid Scintillation Counting

   A. Setting counter

      Packard Tri-Carb liquid scintillation Spectro-
meter Model 3003 with automatic IBM read-out was used
throughout the 125I-UDR uptake studies. A standardization
curve for 125I-UDR of counts/minute vs percentage gain
was plotted using window settings between 50 and 1000 volts
(see Fig. 15). The gain was set at 17%. A background
count of 55 counts/minute was subtracted. Automatic in-
ternal standardization was used throughout to ensure homo-
geneity of experimental conditions.

   B. Preparation of 125I-UDR cell samples for culture

      Duplicate cultures containing 5.00 μc/ml 125I-
UDR (Specific Activity: 1616 mc/mm) purchased from the
Radio-chemical Centre, Amersham, England) and 4 μg/ml
carrier IUDR were prepared in 150 ml prescription bottles
(in total 18 bottles). Each contained 9 ml of cell sus-
pension (10 X 10^4 cells/min. At 12 hour intervals,
after an initial five days of incubation, starting at 0
hour, 0.5 ml of 0.25% trypsin was added to duplicate cul-
tures for sufficient time to release the cells from the
glass surface. The cells were centrifuged at 1500 rpm for
15 minutes. The supernatant was poured off, collected, and
10 ml of Medium "199" was added. Cells were resuspended
in 19.5 ml of Medium "199" containing 4 μg/ml carrier IUDR
and 0.5 ml trypsin solution (for control purposes).

C. Preparation of 125I-UDR cell samples for counting

20 ml plastic scintillation vials (Packard) were
used for counting. The scintillation fluid contained 0.3%
PPO (Packard PPO-2, 5-Diphenyloxazole), and 0.01% POPOP
(Packard Dimethyl POPOP-1, 4-bis-2-) (4-methyl-5-phenyl-
oxazolyl-Benzene) in toluene (Bain and Lowenstein, 1965).
Each vial was prepared as follows (Bell and Hayes, 1957):

- 16.6 ml - 98% ethanol,
- 3.0 ml - scintillation fluid,
- 0.4 ml - cell or supernatant suspension,
- 0.5 gm - Packard Thixotropic Gel Powder.

Vials were capped, designated appropriately,
stored 24 hours and counted for 1 minute.

X. Radioautography

A. Preparation of cultures

Cultures containing IUDR and 125I-UDR were pre-
pared as described under liquid scintillation counting.
Twenty duplicate Leighton tube cultures were set up to
allow for cell attachment. After allowing for cell attach-
ment, the medium was changed and half of the cultures
received 4.5 µc/ml 125I-UDR plus 4 µg/ml IUDR only. The
cultures which had 125I-UDR were removed from incubation
at 24 hour intervals following change of medium (0 hours),
fixed in a solution of glacial acetic acid and methanol
1:3, and stained using the Hematoxylin-Eosin method (see
p. 2). The cover slips were mounted in an inverted posi-
tion on regular glass slides for radioautography. Medium
supplemented with 4.5 µc/ml 125I-UDR and 4.0 µg/ml carrier
IUDR was added to the second series of cultures after five
days of incubation (prior to the medium supplement the
cultures had been grown in the presence of 4 µg/ml IUDR,
see Methods). Cultures were taken off after re-incubation
at 24 hour intervals and stained. Since the first attempt

resulted in an overexposure, a second experiment was set
up. This differed from the first one in the use of 0.5
μc/ml 125I-UDR and 4 μg/ml carrier IUDR as a medium supple-
ment.

B. Preparation of radioautographs

In preparation for radioautography the slides
were exposed to distilled water for 1 hour. The emulsion
used was Kodak Nuclear Track Emulsion, Type NTB^3. This
was diluted 1:1 with distilled water, placed in a coplin
jar and prewarmed in a water bath set at 42°C. The
slides were immersed in the emulsion for 4 to 5 seconds.
Before the slides were put on a drying rack, excess
emulsion was allowed to drip off. After drying, the
slides were placed in a light-tight box. Exposure times
of seven days and ten days were used.

C. Development of radioautographs

After appropriate exposure, the slides were
developed in Dektol (Kodak) for two minutes and rinsed
once in water. Fixation was carried out for 2 to 5
minutes in acid fixer (Kodak). Twenty minutes of rinsing
in water was followed by a final rinse in distilled water,
after which the slides were left to dry.

XI. Photography of Radioautographs and Slides

Microphotographs were made with a Leitz Ortho-
plan microscope, with phase contrast optics and a Leitz
35 mm automatic camera. The light source was a Leitz
Xenon lamp. High contrast Kodak film developed in Kodak
D76 developer, or Adox KB14 film, developed in Kodak
Microdol-X, was used. The enlargements were printed on a
suitable grade of Kodak paper.

RESULTS

I. X-irradiation Damage to Calf Thyroid Cells In Vitro

    A. Cytological consequences of x-irradiation:
        Dose relationships

      Primary diploid monolayer cultures of calf thyroid cells were established for five days and then stained and cytologically examined for chromosomal bridge formation (CB) at intervals following x-ray exposure.

      Plates I through XI are photographs of epithelial calf thyroid cells in various stages of mitosis. Examples of chromosome bridge formations in thyroid cells in vitro (the parameter for the assessment of radiation damage used in this thesis) are pictured in Plates VI through XI.

      Evaluation of control and x-irradiated cultures harvested at 12 hour intervals over a four day period, showed a positive correlation between the frequency of CB and radiation dose. Each percentage shown in Tables I and II (see Figs. 1 and 2) was based on the cytological examination of at least 500 cells. Compared to the controls, the irradiated cultures showed an increase in CB at dose levels of 125 and 250R. However, the increase in CB formation following exposure to x-rays is not proportional to the dose. At 500 and 1000R lower values were observed than expected on the basis of the results obtained with doses of 125 and 250R respectively. The highest percentage of CB was obtained following 250R and at the maximum peak interval (approximately three days) was 64% higher than that found in the control. For dose levels of 500 and 1000R, the difference between CB formation in irradiated and control cultures (when compared with the control), does not appear to be significant. The average increase (64%) in CB at a dose level of 250R is significant and highly reproducible (Figs. 1, 2, 6, 10A, 19, and 20B). Because of

TABLE: I

Influences of x-irradiation on thyroid cells <u>in vitro</u>
(expressed as the percentage of cells showing chromosomal
bridges - CB -) at different culture times post irradia-
tion.

Culture time (in hrs.) of cells post irradiation*	Total dose of X-rays				
	Control	125R	250R	500R	1000R
0 hr.	3.60	3.20	3.00	3.20	1.40
12 hr.	3.00	4.20	4.20	4.20	1.20
24 hr.	3.60	4.20	6.00	5.00	.80
36 hr.	4.60	5.60	6.60	5.40	.80
48 hr.	6.20	7.80	7.20	5.60	2.80
60 hr.	5.20	8.00	12.20	5.20	4.00
72 hr.	5.60	7.20	15.60	4.20	6.60
84 hr.	7.60	7.20	17.20	3.40	5.80
96 hr.	7.20	6.20	7.60	3.80	5.00

* X-irradiation was carried out on cells initially grown
  for 5 days after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

TABLE: II

Influence of x-irradiation on thyroid cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) at different culture times post irradiation.

Culture time (in hrs. of cells post irradiation*	Total dose of x-rays				
	Control	125R	250R	500R	1000R
0 hr.	1.58	1.38	1.58	1.57	1.38
24 hr.	2.58	3,59	3.94	3.74	2.39
48 hr.	3.52	5.24	8.94	4.38	3.55
72 hr.	4.40	8.56	16.00	6.58	6.41
96 hr.	4.57	5.57	7.50	4.18	3.79

* X-irradiation was carried out on cells initially grown for 5 days after which the medium was changed

Note: Each value is based on a count of at least 500 cells.

this, 250R of x-irradiation was chosen for all further
experiments.

    B.   Growth

        Cell multiplication on growth studies in irrad-
iated (250R) and control cultures are shown in Tables
III and V (see Figs. 3 and 5). Figure 3 represents
growth data of thyroid cell cultures which were irradiated
after three days of initial incubation. Figure 5, on the
other hand, pictures growth curves of cultures irradiated
after an initial incubation period of five days. From
Figures 3 and 5 it is evident that a change of medium
after three days of initial incubation resulted in a pro-
longed lag of cell growth, while a medium change after
five days showed little or no lag (see Figs. 3 and 5).
Therefore, in all experiments, irradiation treatments and
medium changes were carried out after five days of initial
incubation. It is interesting to note that a dose of 250R
will not result in significant growth depression (expressed
in terms of cell numbers, Figs. 3 and 5).

    C.   Cytological studies on x-irradiated cultures (250R)

        Tables IV and VI (see also Figs. 4 and 6) repre-
sent frequencies of CB formation in the control and 250R
level. The values represented in Figure 4 are based on the
mean of two different experiments. It will be noted that
the maximum CB values are in agreement with those shown in
Figures 1 and 2. Figures 4A and 6A correlate growth and
CB frequencies of control and irradiated cultures after dif-
ferent incubation periods. A positive correlation exists
between growth (in terms of cell number as a result of
mitotic activity) and CB frequency.

TABLE: III

Influence of 250R. x-irradiation on thyroid cells in
vitro (expressed as the number of cells [X 10,000]/ml
medium) at different culture times.

Culture time (in hrs.) of cells post treatment*	Total dose of x-rays	
	Control	250R
0 hr.	3.7	3.7
12 hr.	5.0	3.7
24 hr.	5.0	5.0
36 hr.	5.0	5.0
48 hr.	5.0	5.0
60 hr.	10.0	10.0
72 hr.	10.0	11.5
84 hr.	15.0	18.0
96 hr.	27.0	23.5

* Treatment was carried out on cells initially grown for
3 days after which the medium was changed.

Note: Each value is based on the average of at least 3
counts.

TABLE: IV

Influence of 250R x-irradiation on thyroid cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Total dose of x-rays	
	Control	250R
0 hr.	**1.00	**1.30
12 hr.	**1.50	**1.30
24 hr.	**2.00	**2.20
36 hr.	**3.40	**3.70
48 hr.	**4.10	**5.90
60 hr.	**6.50	**8.40
72 hr.	**5.30	**11.60
84 hr.	**6.40	**15.20
96 hr.	**6.10	**17.90

*   Treatment was carried out on cells initially grown for 3 days after which the medium was changed.

Note: ** Each value is based on a count of at least 500 cells, of two different experiments.

TABLE: V

Influence of 250R x-irradiation on thyroid cells in vitro (expressed as the number of cells [X 10,000]/ml medium) at different culture times.

Culture time (in hrs.) of cells post treatment*	Total dose of x-ray	
	Control	250R
0 hr.	6.0	5.5
12 hr.	6.5	7.0
24 hr.	12.0	9.0
36 hr.	13.0	13.0
48 hr.	13.0	14.0
60 hr.	25.0	24.0
72 hr.	26.0	25.0
84 hr.	27.0	26.0
96 hr.	27.0	26.0

* Treatment was carried out on cells initially grown for 5 days after which the medium was changed.

Note: Each value is based on an average of at least 3 counts.

TABLE: VI

Influence of 250R x-irradiation on thyroid cells in vitro
(expressed as the percentage of cells showing chromosomal
bridges - CB -) at different culture times post irradia-
tion.

Culture time (in hrs.) of cells post irradiation*	Total dose of x-rays	
	Control	250R
0 hr.	3.60	3.20
12 hr.	4.00	4.20
24 hr.	5.00	5.40
36 hr.	6.40	9.40
48 hr.	7.00	12.00
60 hr.	7.60	17.20
72 hr.	8.00	18.20
84 hr.	8.00	11.00
96 hr.	7.20	8.80

* X-irradiation was carried out on cells initially grown
   for 5 days after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

II.   Consequence of Irradiation on IUDR Pre-treated
      Cultures of Calf Thyroid Cells

   A.   Growth

      The effect of 5-iodo-2'-deoxyuridine (IUDR) on
the growth of the thyroid cultures is seen in Tables VII,
VIII and IX (see Figs. 7, 8 and 9).   IUDR concentrations
of 16 µg/ml or higher (incubation period of five days),
was found to significantly depress the total number of
cells (by approximately 30%).   On the other hand, concen-
trations of IUDR between 1 and 4 µg/ml did not influence
growth.   Cultures which were pre-treated with 4 µg/ml
IUDR and subsequently irradiated (250R) also failed to
exhibit significant growth depression.   Hence, concentra-
tions of 4 µg/ml were used for all further experimentation.

   B.   Cytological evidence of the radiosensitizing
        effect of IUDR

      The frequency of CB formation following expo-
sure to 250R of x-rays was found to be increased when
the cultures were pre-treated with IUDR (4 µg/ml)(see
Tables X and XI, also Figs. 10A, 10B, 11 and 11A.   The
number of CB formed was increased from about 6% in control
to about 16% in x-irradiated cultures.   However, following
pre-treatment with IUDR and irradiation, the frequency of
CB formation was about 22%, an increase over irradiation
alone of 38%.   Figure 11 (see also Fig. 11A) represents
a composite graph demonstrating correlation between growth
(in terms of numbers of cells as a result of mitotic
Activity) and CB frequencies after different incubation
periods of pre-treated cultures.

III.   Evidence of IUDR Uptake by Thyroid Cells
   A.   Paper chromatography studies
      It is established (Eidinoff, Cheong and Rich,
1959) that the thymidine analogue, IUDR is incorporated

TABLE: VII

Influence of the concentration of 5-iodo-2'-deoxyuridine (IUDR) on thyroid cells _in_ _vitro_ (expressed as the number of cells [X 10,000]/ml medium) at different culture times post treatment.

Culture time (in hrs.) of cells post treatment*	5-iodo-2'-deoxyuridine concentrations:			
	Control	4 µg/ml	16 µg/ml	64 µg/ml
0 hr.	7.3	7.0	6.8	5.5
12 hr.	8.0	7.3	7.3	5.7
24 hr.	13.5	10.5	10.5	9.0
36 hr.	14.7	14.0	10.7	9.0
48 hr.	16.7	16.7	12.2	10.2
60 hr.	27.0	26.5	18.0	16.0
72 hr.	31.0	28.5	19.0	17.0
84 hr.	31.0	29.0	21.0	19.0
96 hr.	31.0	30.0	24.0	17.5

* Treatment was carried out on cells initially grown for 5 days in medium with or without a IUDR supplement after which the medium was changed.

Note: Each value is based on the average of at least 3 counts.

- 27 -

TABLE: VIII

Influence of the concentration of 5-iodo-2'-deoxyuridine
(IUDR) on thyroid cells in vitro (expressed as the number
of cells [X 10,000]/ml medium) at different culture times
post treatment.

Culture time (in hrs.) of cells post treatment*	5-iodo-2'-deoxyuridine concentrations:				
	Control	1 μg/ml	2 μg/ml	3 μg/ml	4 μg/ml
0 hr.	9.8	9.7	9.7	9.6	9.6
12 hr.	11.0	11.0	10.5	10.3	10.2
24 hr.	13.8	12.8	12.4	12.6	12.2
36 hr.	13.8	13.0	13.0	13.6	13.6
48 hr.	16.4	16.1	16.2	15.0	14.0
60 hr.	22.4	19.0	18.8	18.8	17.0
72 hr.	27.4	26.2	25.5	23.5	23.4
84 hr.	30.5	29.7	28.4	27.8	27.2
96 hr.	31.5	29.6	29.8	29.0	29.0

* Treatment was carried out on cells initially grown for
5 days in medium with or without a IUDR supplement after
which the medium was changed.

Note: Each value is based on the average of at least 3
counts.

TABLE: IX

Influence of x-irradiation on thyroid cells in vitro
(expressed as the number of cells [X 10,000]/ml medium)
treated with (4 µg/ml) or without IUDR at different cul-
ture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments:			
	Control	250R	4 µg/ml IUDR	4 µg/ml IUDR + 250R
0 hr.	5.5	5.6	5.4	**5.6
12 hr.	5.8	6.0	6.2	**6.3
24 hr.	7.8	8.4	7.4	**8.4
36 hr.	10.2	9.8	10.0	**10.3
48 hr.	11.5	10.3	11.0	**11.2
60 hr.	13.2	13.8	12.5	**13.2
72 hr.	20.4	21.2	19.8	**23.1
84 hr.	22.4	21.8	23.6	**23.1
96 hr.	23.4	21.8	23.4	**22.7

* X-irradiation was carried out on cells initially grown
   for 5 days in medium supplemented with (4 µg/ml) or
   without IUDR after which the medium was changed.

** Average of 2 sets of cultures

Note: Each value is based on the average of at least 3
       counts.

TABLE: X

Influence of 250R x-irradiation on thyroid cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) treated with (4 μg/ml) or without IUDR at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments: Control	250R	4 μg/ml IUDR	4 μg/ml IUDR + 250R a)	b)
0 hr.	2.42	2.75	3.17	3.18	2.95
12 hr.	2.40	3.40	3.18	3.82	4.09
24 hr.	3.45	3.47	3.30	4.92	4.96
36 hr.	3.45	4.47	3.60	6.64	6.40
48 hr.	3.96	7.58	3.78	8.38	8.69
60 hr.	3.69	7.61	3.52	10.03	10.09
72 hr.	5.47	9.89	4.96	13.65	14.31
84 hr.	6.32	16.26	5.64	22.94	21.71
96 hr.	6.75	14.17	5.56	13.72	13.43

* X-irradiation was carried out on cells initially grown for 5 days in medium supplemented with (4 μg/ml) or without IUDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

TABLE: XI

Influence of 250R x-irradiation on thyroid cells in vitro
(expressed as the percentage of cells showing chromosomal
bridges - CB -) treated with (4 µg/ml) or without IUDR at
different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Control	4 µg/ml IUDR	4 µg/ml IUDR + 250R
0 hr.	2.40	**2.80	3.00
12 hr.	2.60	**3.00	3.00
24 hr.	2.60	**3.30	3.40
36 hr.	3.40	**3.90	4.60
48 hr.	3.00	**4.20	6.20
60 hr.	4.20	**3.90	9.60
72 hr.	5.40	**6.20	15.00
84 hr.	6.00	**6.30	23.00
96 hr.	6.00	**5.70	22.00

* X-irradiation was carried out on cells initially grown
   for 5 days in medium supplemented with (4 µg/ml) or with-
   out IUDR after which the medium was changed.

** Average of 3 sets of cultures.

Note: Each value based on a count of at least 500 cells.

into newly replicated DNA of virus, bacteria and mammalian cells. Because the calf thyroid cells in vitro constituted and unexplored systems, it was necessary to prove that IUDR uptake occurred in these cells.

Extracted DNA hydrolysates were chromatographed and the respective R_f values for nucleic bases are given in Table XI (see Fig. 12). Figure 12 is a photograph of the chromatogram obtained. In this chromatogram uracil is located at B. Although the extracted DNA employed for "fingerprinting" would be expected to be free of RNA, uracil will be formed as a result of de-iodonation of iodouracil. Parallel chromatograms employing pure nucleic bases were carried out yielding results which are in agreement with those published by Djordjevic and Szybalski (1960).

B. Radioautographic studies

Plates XVIII, XIX, and XX show that 125I-UDR was incorporated into the nucleus of thyroid cells in vitro. Although the mean energy of 125I (0.0208 Mev) does not allow for a short track length, (incorporated 125I gives rise to particle images at considerable distance [6 - 10μ] from the nuclear disintegration), the maximum grain density observed falls well within the region of the cell nucleus.

From Table XIII (see Figs. 13 and 14) it will be noted that approximately 14 to 16 per cent of the cells counted possessed 125I-UDR nuclear labelling. Moreover, it was found that cells pre-treated with unlabelled IUDR five days prior to transfer to a medium supplemented with a mixture of IUDR and 125I-UDR (examined after five, six, seven, eight and nine days of incubation; Fig. 14, Table XIII), react in a similar quantitative fashion (i.e., in terms of nuclear labelling) to that of experiments grown in the presence of a mixture of IUDR and 125I-UDR but harvested after zero, one, two, three and four days of

TABLE: XII

Chromatographic R_f Values

Nucleic Acid Base	First Dimension	Second Dimension
Thymine	.83	.71
Uracil	.74	.66
Iodouracil	.74	.59
Adenine	.58	.58
Cytosine	.58	.40
Guanine	.37	.37

Note: Each R_f value based on the average of 3 independent runs.

TABLE: XIII

Percent cells with 125I-UDR labelled nuclei

Culture time post treatment	Treatments: Control 4 µg/ml IUDR	4 µg/ml IUDR + 0.5 µc/ml 125I-UDR
a) (Zero-5 day cultures)		
0 hr.	0.00	*0.97
24 hr.	0.00	*2.12
48 hr.	0.00	*6.27
72 hr.	0.40	*12.68
96 hr.	0.00	*14.43

TABLE: XIV

b) (5 - 9 day cultures)		
0 hr.	0.20	*2.98
24 hr.	0.34	*4.31
48 hr.	0.00	*5.79
72 hr.	0.00	*15.24
96 hr.	0.18	*15.97

* Average of 2 counts of at least 500 cells each.

Note: Each value based on a count of at least 500 cells.

incubation (see Fig. 13, Table XIII). Although cultures
of different age (zero and five days) were employed in
the above experiments, pre-treatment of thyroid cells with
IUDR and 125I-UDR supplemented medium did not result in
the selection of IUDR-resistant cell lines.

From a theoretical point of view a frequency of
16 per cent of labelled cells is low, since it can be
assumed that the cell system had undergone at least two
rounds of division (see Fig. 7) during its incubation
period of five days. One has to bear in mind however,
that the actual number of radioactive 125I molecules in
the medium is extremely low relative to the number of
unlabelled iodine molecules. It is for this reason that
the labelled cells with regard to their grain count (30-40
grains per cell nucleus) after radioautography, will be dis-
tributed according to a normal distribution curve. Depending
on the grain count class ranges used by the investigator, the
frequency of labelled cells observed reflects either an ob-
servation in the minimum, the modus or the maximum of the
distribution curve.

C. Liquid scintillation counting

The maximum gain settings for liquid scintillation
counting were determined from Table XV (see Fig. 15).
Table XVI (see Fig. 16) indicates that in cultures incu-
bated for 48 hours, 90% of the 125I-UDR was cell-bound.
The counts in the cell at 36 and 48 hours represent extrapo-
lated data based on the counts in the suspension medium
since cellular localization of 125I-UDR may have resulted in
self-absorption of the low energy radiations (consequently,
the resultant light flashes were not registered by the photo-
multiplier tubes of the liquid scintillation spectrometer).

The results of these chromatographic, radioauto-
graphic, and spectrometric studies confirm that IUDR is

TABLE: XV

Scintillation Standardization Data of 125I

Percent gain setting	Counts/min	Window set at 50-1000 volts
0	55	
10	311,631	
16	336,635	
18	338,488	
20	336,849	
22	337,438	
24	335,591	
30	304,409	
40	208,761	

Note: 17% gain setting was chosen as optimal figure.

TABLE: XVI

Radio-activity (in counts/min) of the supernatant and cellular fraction of thyroid cell cultures grown in the presence of a medium supplement of 125I-UDR after different incubation periods.

Culture time (in hrs.) after addition of 125I-UDR	Counts/min				Total counts/min (1a + 2a)
	1) Supernatant Fraction a)	b)	2) Cellular Fraction a)	b)	
Blank	0	0	0	0	0
0 hr. (control)	176,730	176,619	0	0	176,730
12 hr.	62,336	62,284	121,257	121,147	183,593
24 hr.	63,817	63,682	114,194	114,191	178,011
36 hr.	37,834	37,797	101	103	37,934
			*138,796	*138,930	176,730
48 hr.	9,043	9,035	112	112	9,155
			*167,575	*167,583	

* Extrapolated values by subtraction of sum of supernatant fraction count and cellular fraction count from control value.

incorporated into calf thyroid cells in vitro.

IV.   Consequences of X-irradiation on IUDR Pre-treated
      Cultures of Calf Kidney Cells

   A.   Growth

      In order to compare the IUDR effect on growth in
two autologous cell systems, kidney cell growth was eval-
uated at various concentrations of IUDR.   Table XVII (see
Fig. 17) shows that lUDR at a concentratonn of 4 μg/ml
depressed growth by at least 16% after 96 hours of incu-
bation.   Concentrations of IUDR ranging from 8 to 64
µg/ml depressed growth by approximately 30% after 96 hours
of incubation.   Table XVIII (see Fig. 18) indicates that
x-irradiation alone did not have an immediate depressive
effect on kidney cell growth.   However, x-irradiation
coupled with a pre-treatment (4 µg/ml IUDR supplement in
the growth medium) depressed the cell number by 25%.

      The maxima of CB for kidney cells irradiated
with 250R with kidney cells pre-treated with IUDR and
subsequently exposed to 250R, do not coincide due to the
growth depression of kidney cells cultured in the presence
of IUDR.

   B.   Cytological evidence of radiosensitizing effect
        of IUDR on kidney cells

      Plates XII through XVII are photographs of epi-
thelial calf kidney cells in various stages of mitosis.
Tables XX and XXI (see Figs. 20A and 20B) represent values
obtained from two parallel experiments.   Table XIX (see
Fig. 19) represents data obtained from a completely inde-
pendent experiment.   Each experiment was carried out with
a 4 µg/ml supplement of IUDR in the growth medium.   The
graphic representation shows enhancement of CB's when IUDR
pre-treated cultures were subsequently exposed to

TABLE: XVII

Influence of the concentration of 5-iodo-2'-deoxyuridine (as a medium supplement) on kidney cells in vitro (expressed as the number of cells [X 10,000]/ml medium at different culture times post treatment.

Culture time (in hrs.) of cells post treatment*	Control	4 µg/ml IUDR	8 µg/ml IUDR	16 µg/ml IUDR	32 µg/ml IUDR	64 µg/ml IUDR
0 hr.	16.4	16.4	13.0	11.4	11.8	8.8
12 hr.	26.6	20.6	16.4	14.6	14.2	14.2
24 hr.	23.8	21.2	18.6	18.6	16.6	15.6
36 hr.	29.0	21.2	21.0	20.8	20.2	19.6
48 hr.	30.0	26.8	22.2	23.2	20.8	19.8
60 hr.	39.0	32.2	27.0	26.2	25.8	26.0
72 hr.	39.2	32.8	27.2	26.8	26.4	26.6
84 hr.	40.0	33.4	27.6	27.0	26.4	26.8
96 hr.	40.4	33.6	28.0	28.4	28.0	26.8

* Treatment was carried out on cells initially grown for 5 days after which the medium was changed.

Note: Each value is based on the average of at least 3 counts.

TABLE: XVIII

Influence of x-irradiation on kidney cells in vitro
(expressed as the number of cells [X 10,000]/ml medium)
with and without 4 ug/ml IUDR at different culture times
post irradiation.

Culture time (in hrs.) of cells post irradiation	Treatments:		4 µg/ml IUDR
	Control	250R	+ 250R
0 hrs.	16.4	16.0	17.0
12 hrs.	26.6	25.8	20.4
24 hr.	28.8	27.0	23.0
36 hr.	29.0	27.6	23.6
48 hr.	30.0	29.6	25.0
60 hr.	39.0	37.8	29.2
72 hr.	39.2	38.8	30.0
84 hr.	40.0	40.4	31.0
96 hr.	40.4	39.8	31.0

* X-irradiation was carried out on cells initially grown
   for 5 days in medium supplemented with (4 µg/ml) or
   without IUDR after which the medium was changed.

Note: Each value is based on the average of at least 3
       counts.

TABLE: XIX

Influence of x-irradiation on kidney cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) treated with (4 µg/ml) and without IUDR at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments:		4 µg/ml	4 µg/ml IUDR +
	Control	250R	IUDR	250R
0 hr.	2.53	1.99	2.73	2.69
12 hr.	2.58	3.15	2.76	2.80
24 hr.	2.74	4.33	2.96	3.74
36 hr.	3.98	11.48	4.33	7.53
48 hr.	5.26	12.59	4.68	12.15
60 hr.	5.69	15.76	5.88	12.62
72 hr.	4.54	10.95	5.84	19.13
84 hr.	4.13	10.78	4.19	16.66
96 hr.	3.97	10.95	4.57	12.00

* X-irradiation was carried out on cells initially grown for 5 days in medium supplemented with (4 µg/ml) or without IUDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

TABLE: XX

Influence of x-irradiation on kidney cells in vitro
(expressed as the percentage of cells showing chromosomal
bridges - CB -) treated with (4 µg/ml) and without IUDR
at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments: Control	250R	4 µg/ml IUDR	4 µg/ml IUDR + 250R
0 hr.	3.37	3.50	2.93	2.75
12 hr.	3.91	4.73	2.92	3.98
24 hr.	3.74	5.63	3.35	7.37
36 hr.	3.88	11.79	3.40	7.40
48 hr.	4.10	12.74	4.09	11.41
60 hr.	5.38	15.96	5.77	15.47
72 hr.	5.52	15.36	6.71	17.15
84 hr.	6.60	11.46	5.51	17.65
96 hr.	4.74	11.70	4.88	13.53

*   X-irradiation was carried out on cells initially grown
    for 5 days in medium supplemented with (4 µg/ml) or
    without IUDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

TABLE:: XXI

Influence of x-irradiation on kidney cells in vitro
(expressed as the percentage of cells showing chromosomal
bridges - CB -) treated with (4 µg/ml) and without IUDR
at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments: Control	250R	4 µg/ml IUDR	4 µg/ml IUDR + 250R
0 hr·	3.37	3.80	2.93	3.09
12 hr.	3.91	5.05	2.92	3.75
24 hr.	3.74	6.16	3.35	7.22
36 hr.	3.88	10.67	3.40	7.88
48 hr.	4.10	13.40	4.09	10.77
60 hr.	5.38	14.56	5.77	15.10
72 hr.	5.52	15.55	6.71	16.43
84 hr.	6.60	12.37	5.51	19.20
96 hr.	4.74	11.35	4.88	13.81

* X-irradiation was carried out on cells initially grown
  for 5 days in medium supplemented with (4 µg/ml) or
  without IUDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

x-irradiation. When compared with the control, the IUDR
pre-treated and 250R x-irradiated cultures show an
increase in CB frequency of approximately 70% at peak
interval. When the maximum peaks for x-irradiated and
IUDR pre-treated cultures are compared the differential
radiosensitivity in terms of CB frequency amounts to
approximately 8 to 16% (mean value for all experiments
is 12%). Hence the radio-potentiation of IUDR for kidney
cells, as based on the CB frequency, amounts to approx-
imately one-third that for thyroid cells (see Discussion).

V. Consequences of X-irradiation on ICDR Pre-treated
     Cultures of Thyroid Cells

     A. Growth

          Cultures were grown in various concentrations
of ICDR supplemented medium in order to compare the
results with those found for cultures grown in IUDR
supplemented medium. On the basis of cell growth studies,
ICDR appears to be less toxic to thyroid cells than IUDR.
No significant depression of cell growth was evident at
concentratons as high as 64 µg/ml of ICDR (see Table XXII,
Fig. 22). In fact, this 64 µg/ml of ICDR appeared to
have a stimulating effect on cell growth. An absence of
growth depression of thyroid cells was noted not only for
cultures treated with ICDR, but also for such cultures
subsequently irradiated with 250R (see Table XXIII, Fig. 23).

     B. Cytological evidence of the radiosensitizing
          Effect of ICDR on thyroid cells

          The incidence of CB in cultures treated with
either 4 µg/ml ICDR (see Table XXV, Fig. 25) or 64 µg/ml
ICDR (see Table XXIV, Fig. 24) and subsequently x-irradiated
with 250R was similar. Table XXIV (see Fig. 24) represents
values from an independent experiment in which a medium
supplemented with 64 µg/ml ICDR was used. As can be seen,

TABLE: XXII

Influence of the concentration of 5-iodo-2'-deoxycytidine
(as a medium supplement) on thyroid cells in vitro (expressed
as the number of cells [X 10,000]/ml medium) at different
culture times post treatment.

Culture time (in hrs.) of cells post treatment*	Treatments: ICDR concentrations			
	Control	4 µg/ml	16 µg/ml	64 µg/ml
0 hr.	16.0	16.0	15.6	16.4
12 hr.	16.4	16.4	16.4	17.0
24 hr.	17.4	17.6	18.0	22.5
36 hr.	25.0	25.0	25.2	25.2
48 hr.	28.4	29.6	29.0	33.6
60 hr.	28.6	29.8	30.0	36.6
72 hr.	32.0	32.4	31.2	37.4
84 hr.	38.4	37.8	38.4	38.0
96 hr.	39.4	38.0	38.6	37.4

*   Treatment was carried out on cells initially grown for
    5 days after which the medium was changed.

Note: Each value is based on the average of at least 3
        counts.

TABLE: XXIII

Influence of x-irradiation on thyroid cells in vitro
(expressed as the number of cells [X 10,000]/ml medium)
treated with 64 µg/ml ICDR.

Culture time (in hrs.) of cells post treatment*	Treatments: ICDR concentration and irradiation		
	Control	64 µg/ml ICDR	64 µg/ml ICDR + 250R
0 hr.	15.4	16.4	16.0
12 hr.	15.6	18.2	17.4
24 hr.	18.8	24.2	24.0
36 hr.	26.4	26.4	26.6
48 hr.	28.0	28.2	27.8
60 hr.	29.4	28.6	30.4
72 hr.	37.8	36.6	38.2
84 hr.	38.0	39.0	38.4
96 hr.	38.0	38.8	38.6

* Treatment was carried out on cells initially grown for
  5 days with and without 64 µg/ml ICDR after which the
  medium was changed.

Note: Each value is based on the average of at least 3
      counts.

TABLE: XXV

Influence of x-irradiation on thyroid cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) treated with 4 μg/ml ICDR at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments: ICDR concentrations and irradiation		
	Control	4 μg/ml ICDR	4 μg/ml ICDR + 250R
0 hr.	2.29	2.84	3.08
12 hr.	2.58	3.28	4.76
24 hr.	3.19	3.83	5.52
36 hr.	3.79	4.46	5.61
48 hr.	4.23	4.79	11.68
60 hr.	6.04	.585	12.90
72 hr.	5.81	7.03	19.25
84 hr.	7.81	7.96	22.73
96 hr.	5.76	8.78	16.66

*   X-irradiation was carried out on cells initially grown for 5 days in medium supplemented with or without 4 μg/ml ICDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells. The irradiated (250R) control has been omitted.

TABLE: XXIV

Influence of x-irradiation on thyroid cells in vitro
(expressed as the percentage of cells showing chromosomal
bridges - CB -) treated with 64 µg/ml ICDR at different
culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments:		
	Control	64 µg/ml ICDR	64 µg/ml ICDR + 250R
0 hr.	1.76	1.90	*2.00
12 hr.	2.95	2.35	*4.30
24 hr.	4.63	4.52	*8.10
36 hr.	5.96	6.20	*9.60
48 hr.	7.02	6.75	*11.10
60 hr.	7.05	7.23	*17.20
72 hr.	7.60	7.33	*23.10
84 hr.	8.71	7.63	**23.60
96 hr.	8.00	7.85	**21.30

* X-irradiation was carried out on cells initially grown
for 5 days in medium supplemented with or without
64 µg/ml ICDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.
* Average of 3 counts of at least 500 cells for each value.
** Average of 6 counts of at least 500 cells for each value.
The irradiated (250R) control has been omitted.

no further enhancement of radio-potentiation of the thyroid
cell is evoked by ICDR concentrations between 4 and 64
µg/ml. Figures 24A and 25A are composite graphs relating
growth under different environmental conditions and in-
cubation time with the frequency of CB in cultures treated
in a similar way. For all conditions studied, a correla-
tion between growth (expressed as the number of cells/ml
medium) and the number of CB formed, was observed.

VI. Consequence of X-irradiation and IUDR-ICDR Pre-treated
     Cultures on Calf Thyroid Cells

     A. Growth

          As has already been noted in preceding experiments
IUDR and ICDR as independent medium supplements equally
potentiate x-irradiation induced CB formation. Since the
upper limit of IUDR toxicity (4 µg/ml) does not coincide
with the upper limit of ICDR toxicity it is feasible to
explore any synergism in the effect of these two compounds.
In other words, a cooperative effect by a combination of
IUDR and ICDR might raise the CB frequency above the level
produced by either compound. In this particular model it
is assumed that the incorporation modes of ICDR and IUDR
differ. The lack of a synergistic effect would indicate
that these two compounds act as a single entity, despite
their chemical differences.

          Table XXVI (see Fig. 26) is the growth data
from an experiment in which a 4 µg/ml IUDR and 4 µg/ml
ICDR combination was used as a growth medium supplement.
Cell growth is not impaired when a mixture of IUDR and
ICDR was used whether or not combined with x-irradiation.

     B. Cytological evidence of the radiosensitizing
         effect of a combined IUDR - ICDR supplemented
         medium

          The combined use of IUDR and ICDR in the medium
prior to irradiation did not enhance radiation induced CB

TABLE: XXVI

The influence of 250R x-irradiation on thyroid cells
in vitro (expressed as the number of cells [X 10,000]/ml
medium) treated with or without a mixture of IUDR and
ICDR at different culture times post irradiation.

Culture time (in hrs.) of cells post irradiation*	Treatments: IUDR + ICDR concentrations and irradiation			
	Control	250R	4 µg/ml IUDR + 4 µg/ml ICDR	4 µg/ml IUDR + 4 µg/ml ICDR + 250R
0 hr.	16.4	16.0	16.2	16.4
12 hr.	18.0	17.8	17.6	18.0
24 hr.	18.2	18.4	18.4	18.4
36 hr.	26.8	27.8	27.2	27.0
48 hr.	29.4	28.6	27.4	28.8
60 hr.	29.6	29.0	29.8	28.9
72 hr.	39.0	37.0	34.8	32.0
84 hr.	39.4	38.8	37.8	37.4
96 hr.	39.7	39.0	38.0	37.8

* X-irradiation was carried out on cells initially grown
for 5 days in medium supplemented with or without a mixture
of IUDR and ICDR (4 µg/ml of each) after which the medium
was changed.

Note: Each value is based on the average of at least 3
counts.

frequency, when compared with the effect of each of the analogues alone (see Fig. 27). Figure 27A is a composite graph, relating cell growth under different environmental conditions and after different incubation periods, with CB frequencies obtained under similar conditions. No explanation can be offered for the consistently higher CB formation frequency of the combined IUDR and ICDR control when compared to the non-supplemented control (Table XXVII).

VII. Consequences of Continuous 131I-irradiation on Calf Thyroid Cells

A. Growth

Experiments were carried out to determine the effects on growth of an accumulated dose from 131I (approximately 300 rads after four days of incubation). Table XXVIII (see Fig. 28) represents growth (in terms of cell numbers) after different periods of incubation. A slight depression in growth occurred after 300 rad irradiation (131I) and in cultures pre-treated with IUDR and subsequently irradiated with a dose of 300 rad (131I).

B. Absence of the radiosensitizing effect of IUDR on calf thyroid cells in vitro under continuous irradiation

Table XXIX (see Fig. 29) represents cytological data (in terms of CB) derived from a single experiment. The results indicated that the incorporation of the thymidine analogue IUDR had little or no radiosensitizing effect when used in combination with continuous irradiation. The magnitude of CB formation after continuous irradiation damage is almost equal to that obtained with x-irradiation. From a comparison of appropriate curves (Figs. 29, 30A and 30B) it appears that IUDR pre-treatment does not contribute to the frequency of CB formation in cultures subsequently exposed to 300 rad continuous irradiation (131I).

TABLE: XXVII

The influence of 250R x-irradiation on thyroid cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) treated (i) with or without a medium supplement of 4 µg/ml IUDR; (ii) with a medium supplement of 4 µg/ml ICDR; or (iii) with a medium supplement of a mixture of 4 µg/ml IUDR ICDR.

Culture time (in hrs.) of cells post irradiation*	Treatments: IUDR and ICDR concentrations and Irradiation					
	Control	250R	4 µg/ml IUDR + 250R	4 µg/ml/ICDR + 250R	4 µg/ml/ICDR + 4µg/ml ICDR	4 µg/ml/ICDR 4 µg/ml IUDR + 4 µg/ml ICDR + 250R
0 hr.	4.50	4.35	5.11	4.34	7.15	*7.62
12 hr.	4.70	4.78	4.19	4.86	7.19	*8.41
24 hr.	5.11	5.03	5.45	5.66	8.59	*9.61
36 hr.	5.85	12.45	13.00	13.51	9.02	*14.99
48 hr.	6.38	13.88	14.42	14.65	9.09	*15.92
60 hr.	6.34	14.93	15.55	14.90	9.12	*18.26
72 hr.	7.66	17.15	19.48	19.52	10.00	*22.07
84 hr.	-	17.46	20.78	22.52	11.85	*21.91
96 hr.	9.37	16.69	-	21.24	10.77	*15.53

* X-irradiation was carried out on cells initially grown for 5 days in medium with or without analogue supplement after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

* Value based on the average of 3 counts of 500 cells.

TABLE: XXVIII

Influence of continuous 131I-irradiation on thyroid cells in vitro (expressed as the number of cells [X 10,000]/ml medium) treated with (4 μg/ml) or without IUDR after different incubation periods.

Culture time (in hrs.) of cells post medium change and initiation of 131I-irradiation*	Treatments: IUDR and ICDR concentrations and irradiation.			
	Control	4 μg/ml IUDR	300 rad-131I (irradiation given over 4 days)	4 μg/ml IUDR + 300 rad-131I (irradiation given over 4 days)
0 hr.	7.8	7.8	7.4	7.6
12 hr.	8.0	8.2	7.8	8.0
24 hr.	11.0	9.6	11.2	9.8
36 hr.	12.8	12.0	11.4	11.0
48 hr.	12.8	12.2	11.2	11.2
60 hr.	15.0	16.0	14.4	14.0
72 hr.	19.8	18.6	16.8	16.0
84 hr.	19.8	18.6	17.8	16.2
96 hr.	20.8	19.0	17.8	17.2

* Treatment of the cells with 131I-irradiation followed an initial period of growth for 5 days in medium supplemented with or without IUDR after which the medium was changed.

Note: Each value based on the average of at least 3 independent counts.

TABLE: XXIX

Influence of continuous 131I-irradiation on thyroid cells
in vitro (expressed as the percentage of cells showing
chromosomal bridges - CB -) treated with (4 µg/ml) or
without IUDR after different incubation periods.

Culture time (in hrs.) of cells post medium change and initiation of 131I-irradiation*	Treatments:			
	Control	4 µg/ml IUDR	300 rad- 131I	4 µg/ml IUDR + 300 rad- 131I
0 hr.	2.39	1.99	2.94	1.97
12 hr.	2.98	2.38	2.76	2.34
24 hr.	3.10	2.38	3.95	4.45
36 hr.	3.14	3.19	4.80	6.69
48 hr.	3.57	3.38	4.99	7.00
60 hr.	3.55	4.14	6.86	9.80
72 hr.	6.61	4.55	-	-
84 hr.	5.98	4.71	14.27	15.90
96 hr.	5.13	5.61	-	14.94

* Treatment of the cells with 131I-irradiation followed
and initial period of growth for 5 days in medium
supplemented with or without IUDR after which the
medium was changed.

Note: Each value based on a count of at least 500 cells.

Tables XXXA and XXXB (see Figs. 30A and 30B) represent CB values derived from an independent experiment in duplo. Although the maximum CB frequency is slightly higher than the one reported in Figure 29, the results of the experiment concur with the observation reported above. In other words, IUDR does not potentiate continuous 131I-irradiation damage when compared to a single dose of x-irradiation combined with IUDR pre-treatment. From Figures 30A and 30B it will be noted, that the maximum peaks of CB due to x-irradiation and x-irradiation coupled with IUDR pre-treatment, are in agreement with the results reported in previous experiments. Figure 31 is a composite graph of figures 28 and 29, showing a positive correlation between CB frequency and cell multiplication.

VIII. Effect of Various Treatments on Clone Forming Ability

An experiment in triplicate was carried out involving the major treatments as reported in previous experiments (see Fig. 32). The parameter of this experiment consisted of the frequency of clones (i.e. two or more cells) developed in culture after various incubation intervals. Clone size (usually 8 cells or more) as a papameter was not considered.

Table XXI (see Fig. 32) summarizes the findings derived from these experiments. Attention is drawn to the 96 hours interval, where the largest differences in clone numbers was observed. The number of clones in control, IUDR and ICDR treated cultures (120, 108, 109 clones respectively) were similar (see Fig. 32). The clone number in x-irradiated IUDR and ICDR cultures (64, and 68 respectively) were also similar, but lower than those found in the non-irradiated control cultures. Cultures continuously irradiated for four days with 131I with and without IUDR pre-treatment (60 clones respectively) showed a similarly

TABLE: XXX (A and B)

Comparison between the influences of 300 rad 131I-irradiation and 250R x-irradiation of thyroid cells in vitro (expressed as the percentage of cells showing chromosomal bridges - CB -) and treated with (4 μg/ml medium) or without IUDR after different incubation periods.

Treatments:

Culture time (in hrs.) of cells post x-irradiation or post 131I-irradiation onset*	Control	250R	4 μg/ml IUDR	4 μg/ml IUDR + 250R	4 μg/ml 300 rad-131I		4 μg/ml IUDR + 300 rad-131I	
					a)	b)	a)	b)
0 hr.	1.94	1.98	1.59	1.77	2.17	1.96	2.39	2.17
12 hr.	2.53	2.85	3.13	2.99	2.97	3.16	3.80	3.38
24 hr.	4.05	4.35	3.36	5.15	4.52	4.18	4.99	3.99
36 hr.	4.33	7.00	3.79	7.95	6.71	7.03	8.89	8.08
48 hr.	4.55	7.88	4.18	7.78	7.35	7.46	8.15	8.16
60 hr.	5.12	10.37	3.94	8.96	14.99	14.48	9.01	9.10
72 hr.	5.56	16.46	5.77	22.68	12.30	11.11	13.28	12.57
84 hr.	6.73	14.54	6.86	16.01	18.32	18.62	18.36	18.38
96 hr.	6.52	14.17	6.37	15.88	15.87	17.18	17.65	17.36

* Radiation treatment was carried out on cells initially grown for 5 days in medium supplemented with (4 μg/ml medium) or without IUDR after which the medium was changed.

Note: Each value is based on a count of at least 500 cells.

TABLE: XXXI

Comparison of the effects of various treatments on thyroid cells in vitro (expressed as the number of clones/10 predetermined fields) after different incubation periods.

Culture time (in hrs.) after seeding	Treatments: Control	250R	4 µg/ml IUDR	4 µg/ml IUDR + 250R	4 µg/ml ICDR	4 µg/ml ICDR + 250R	300 rad-131I	4 µg/ml IUDR + 300 rad-131I
0 hr.	**0	**0	**0	**0	**0	**0	**0	**0
24 hr.	48	37	51	42	36	44	42	32
48 hr.	85	70	77	83	65	80	78	80
72 hr.	99	79	93	71	68	64	52	70
96 hr.	120	74	108	64	109	68	60	60
120 hr.	92	80	88	69	91	72	50	68

Radiation treatment was carried out on cells initially grown for 5 days in medium supplemented with 4 µg/ml IUDR or ICDR or without IUDR or ICDR, after which the medium was changed.

** At zero hour no cell attachment.

Note: Each value is based on the average of 3 cultures. The values were not based on clone size (that is the number of cells in the clone, two and over).

clustered pattern when plotted at approximately the same
level as was obtained for the x-irradiated IUDR and ICDR
pre-treated cultures (64 and 68) clones respectively). The
250R cultures showed a value somewhat intermediate to other
experimental results (74 clones).

The results reported above are in relatively good
agreement with cytological data. No intentional data with
regard to plating efficiency of irradiated cells vs. non-
irradiated cells were collected. However, from previous growth
experiments (see Figs. 5, 9, 23, 28) it can be deducted that
the plating efficiency is not affected by the radiation levels
employed.

FIG. 1

FIG. 2

No. Cells / ml (X 10,000)

HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE. (MEDIUM CHANGE AFTER 3 DAYS)

FIG. 3

Control

250 r

FIG. 4

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE (MEDIUM CHANGE AFTER 3 DAYS CULTURE)

Control
250 r
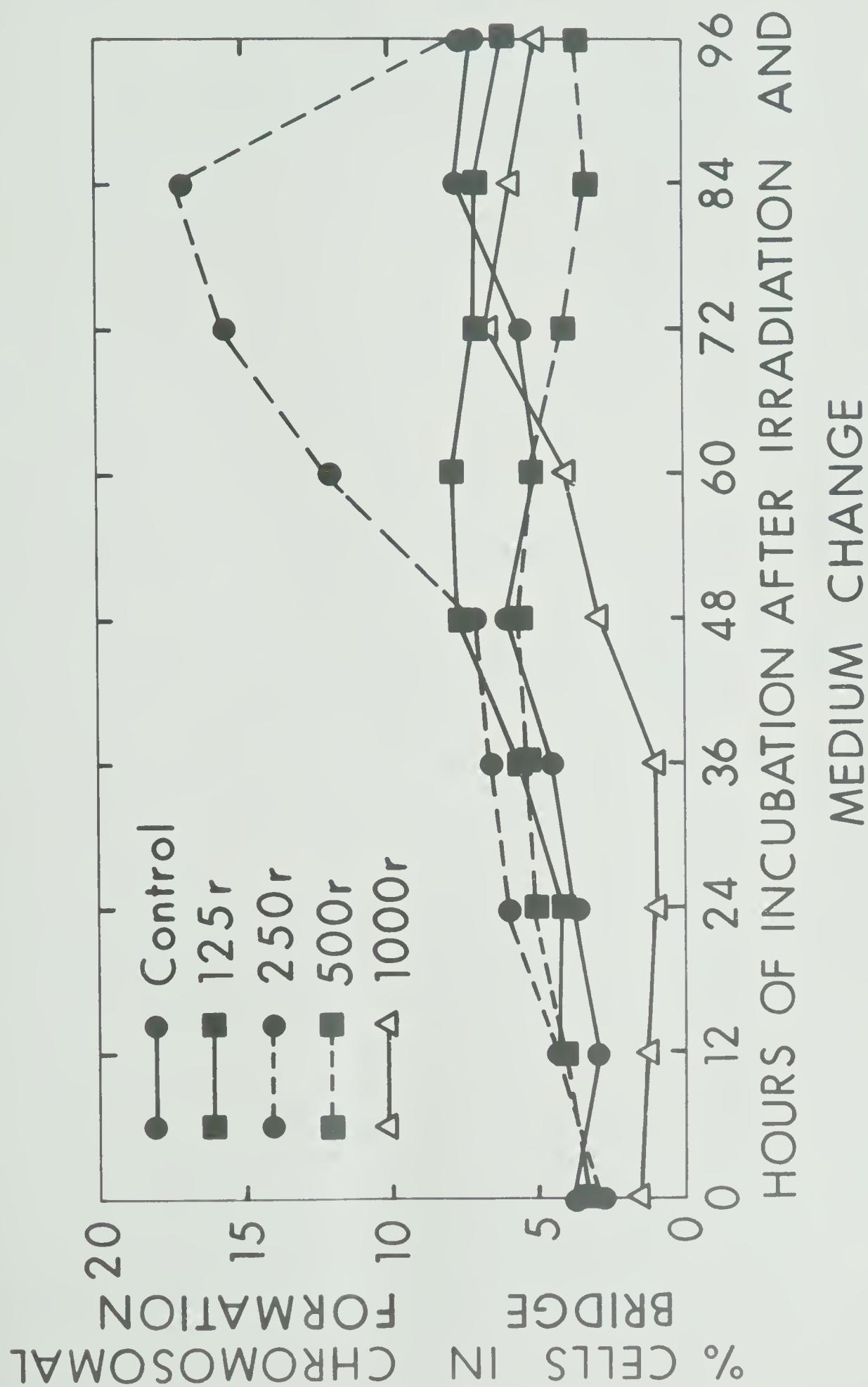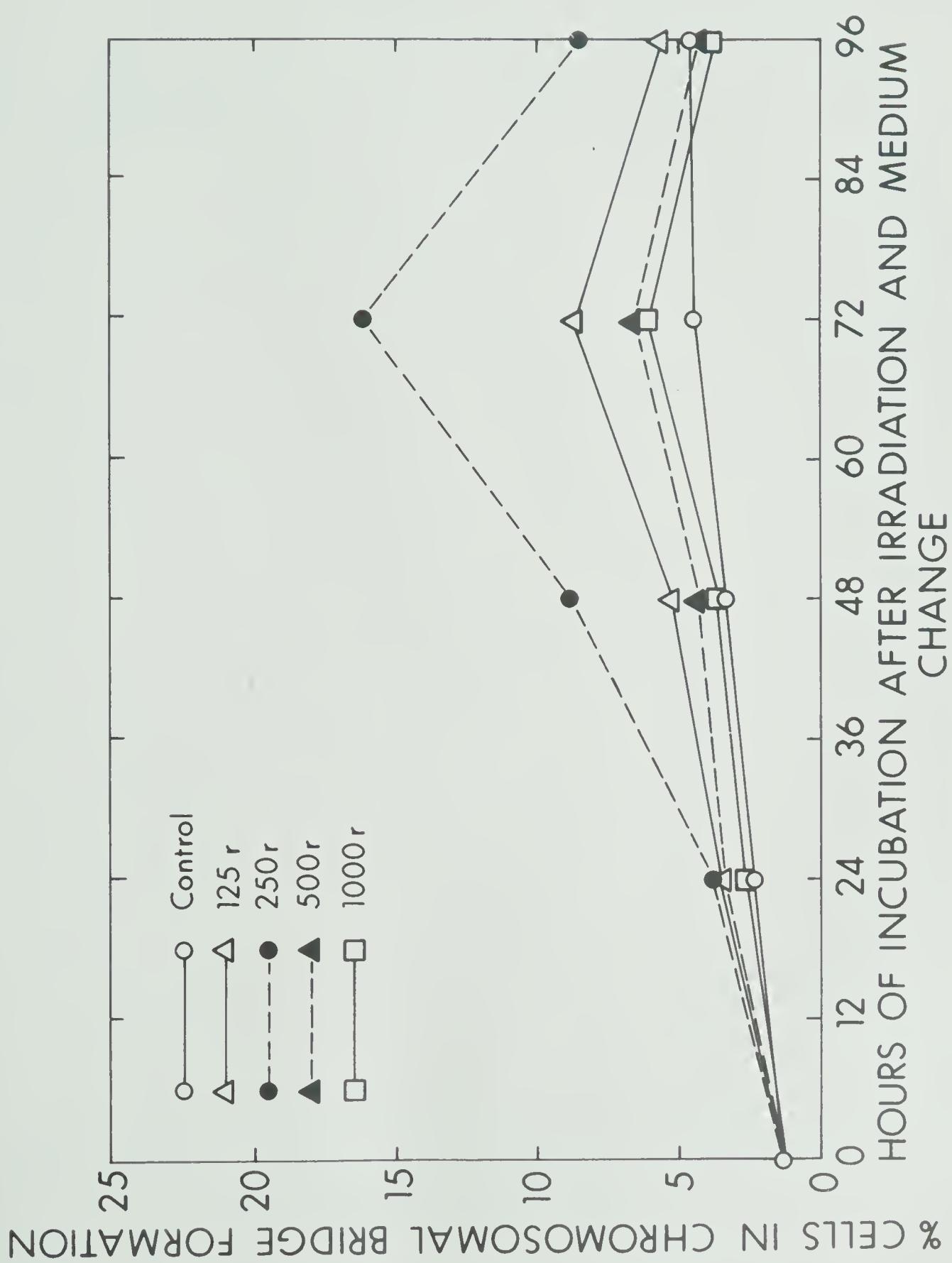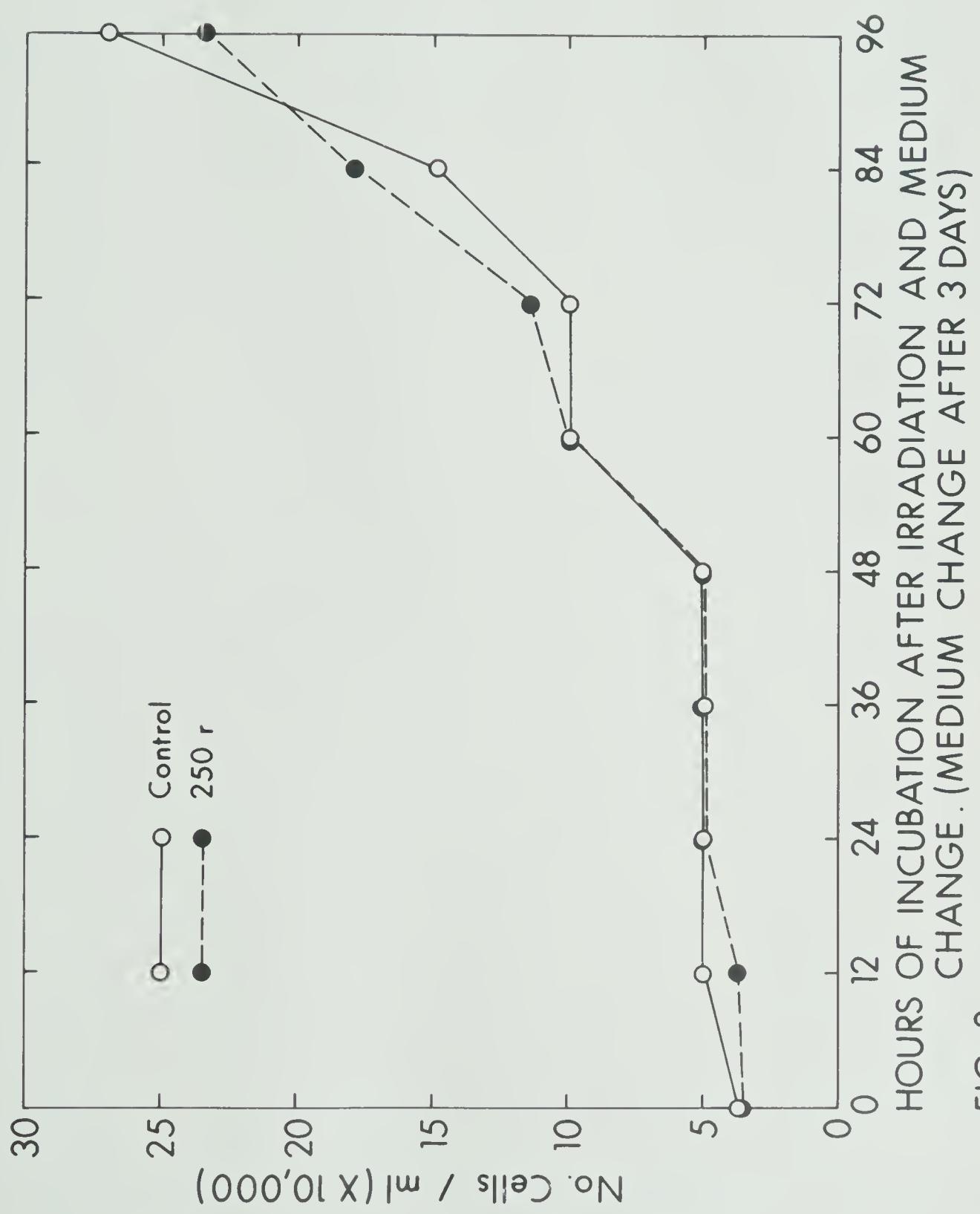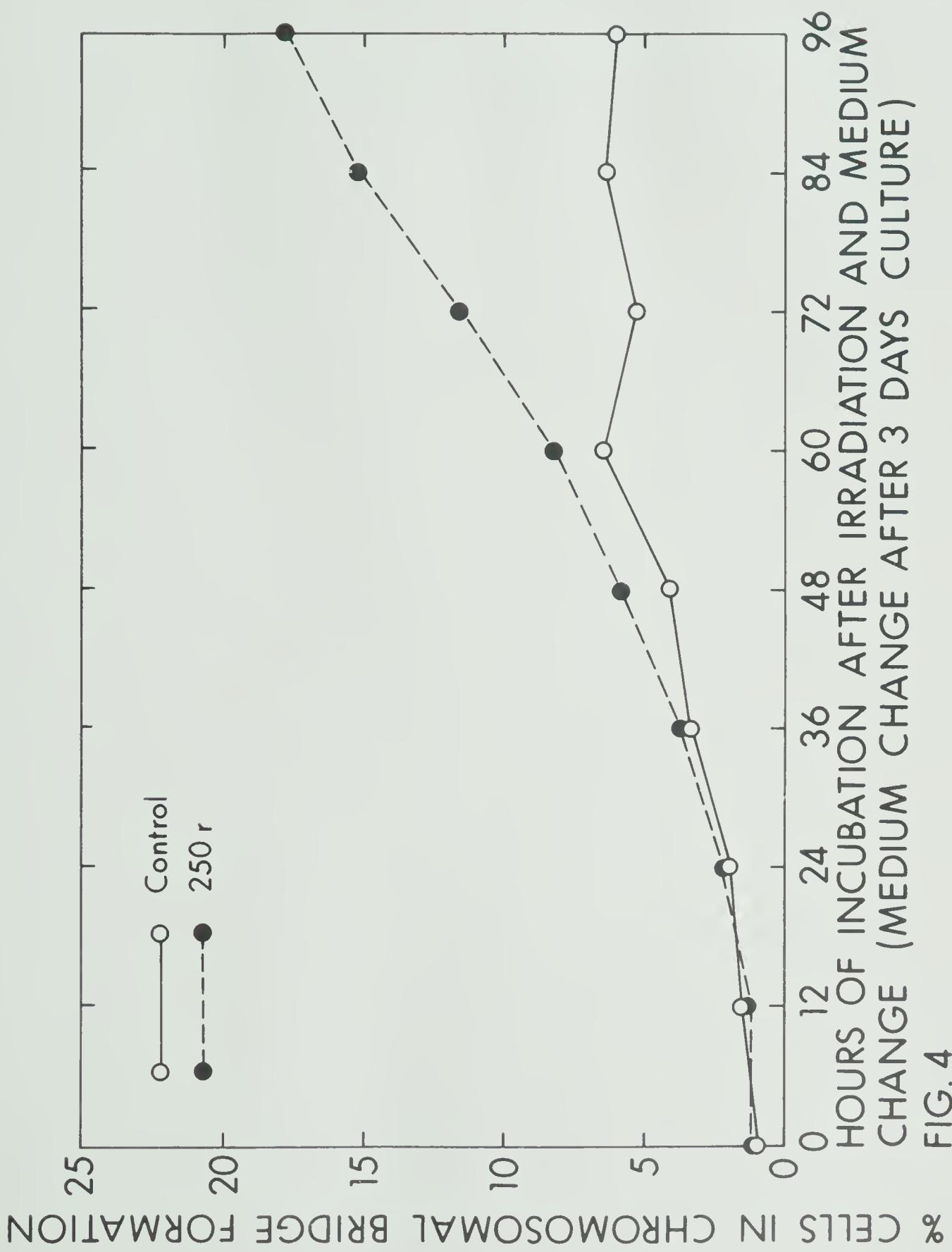
Fig. 4A. A composite graph of Figs. 3 and 4 demonstra-
ting the dependence of chromosomal bridge
formation on mitotic activity.

No. Cells /ml (x10,000)

Control △ Growth
250 r ▲

Control ○ CB
250 r ●

HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE (MEDIUM CHANGE AFTER 3 DAYS CULTURE)

FIG. 4A

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

HOURS OF INCUBATION AFTER IRRADIATION
AND MEDIUM CHANGE
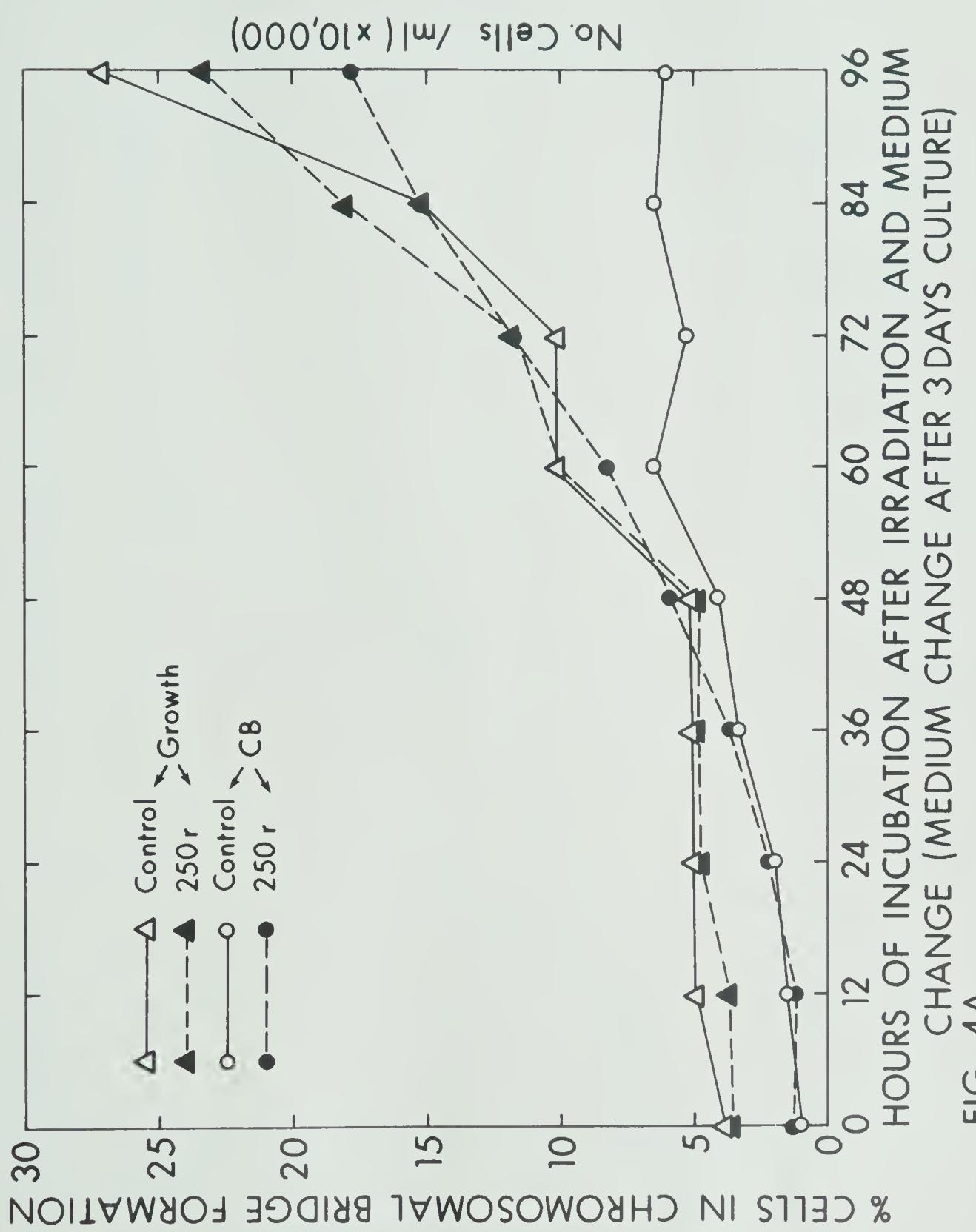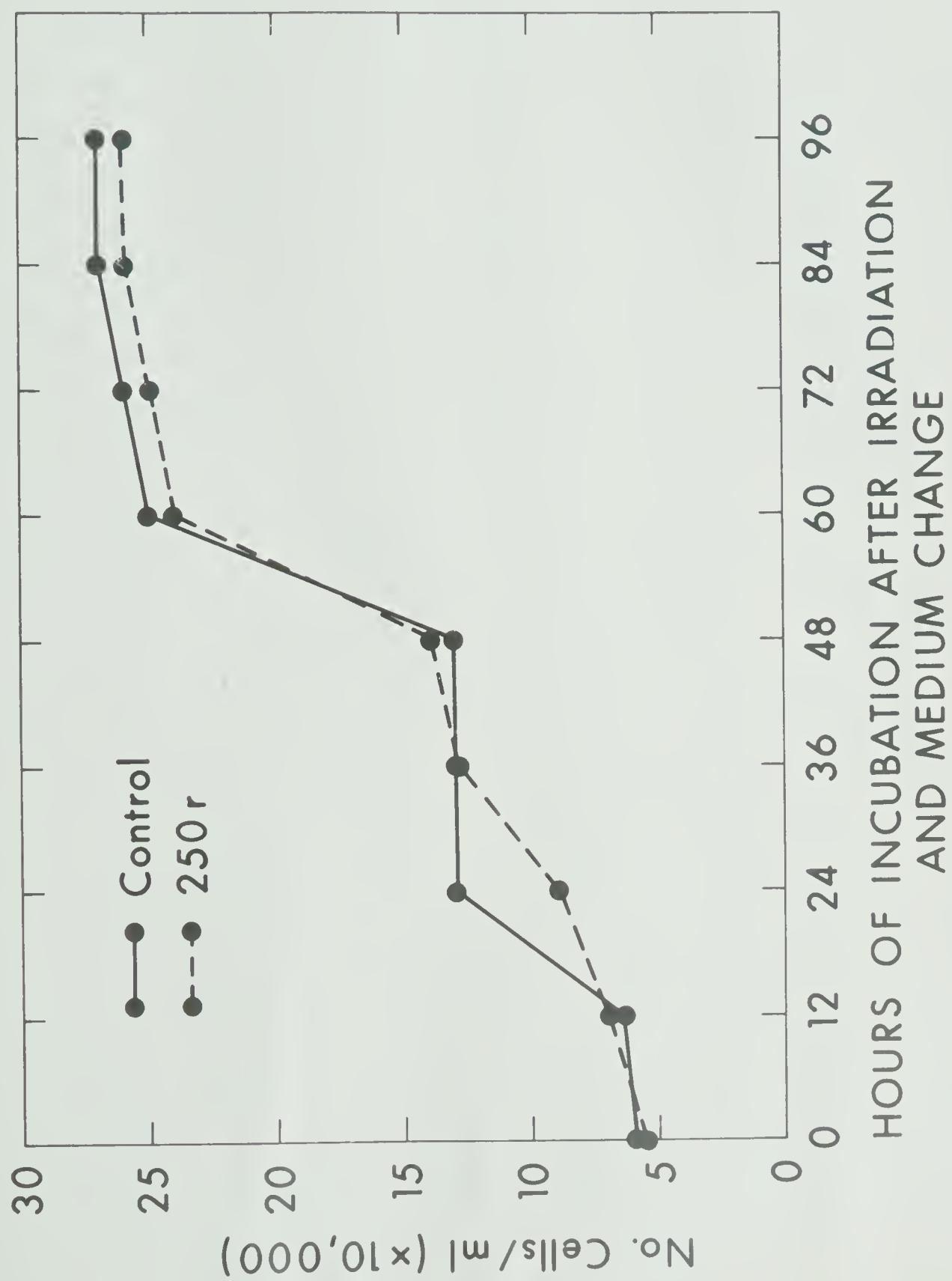
FIG. 5
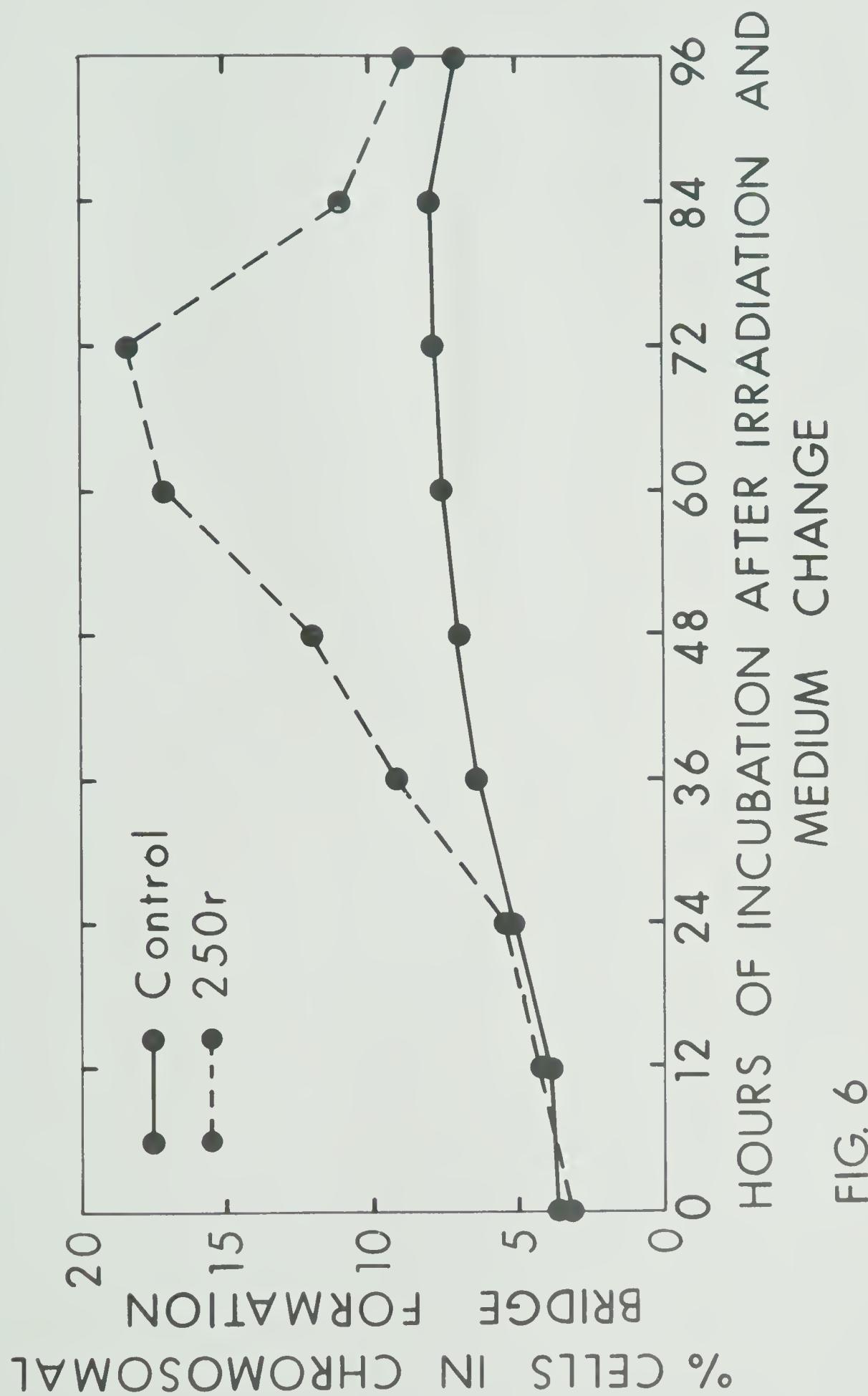
FIG. 6

Fig. 6A. A composite graph of Figs. 5 and 6 demonstra-
ting the dependence of chromosomal bridge
formation on mitotic activity.
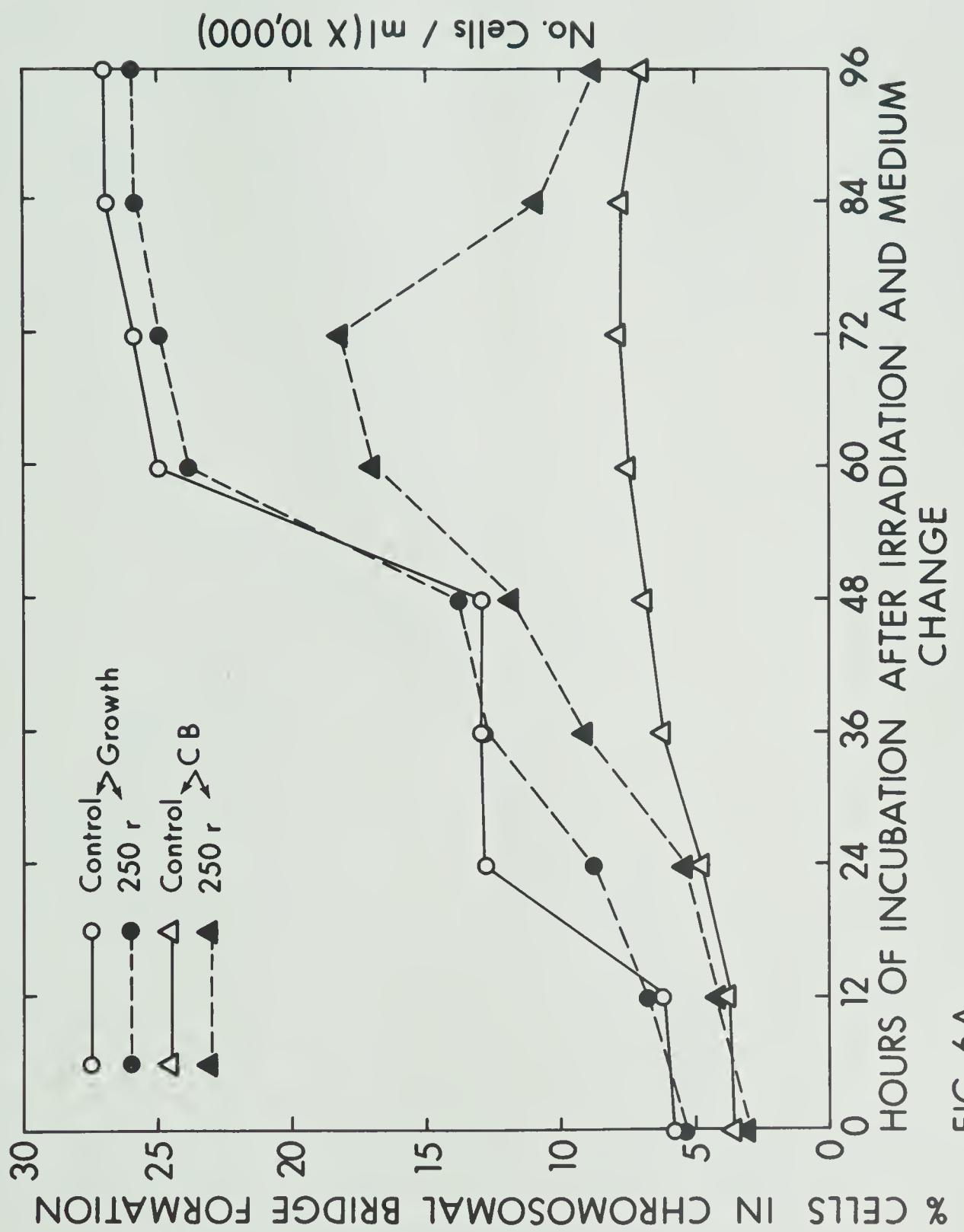
FIG. 6A

FIG. 7 HOURS OF INCUBATION AFTER MEDIUM CHANGE

No. Cells/ml (×10,000)

Control
4μg/ml IUDR
16μg/ml IUDR
64μg/ml IUDR
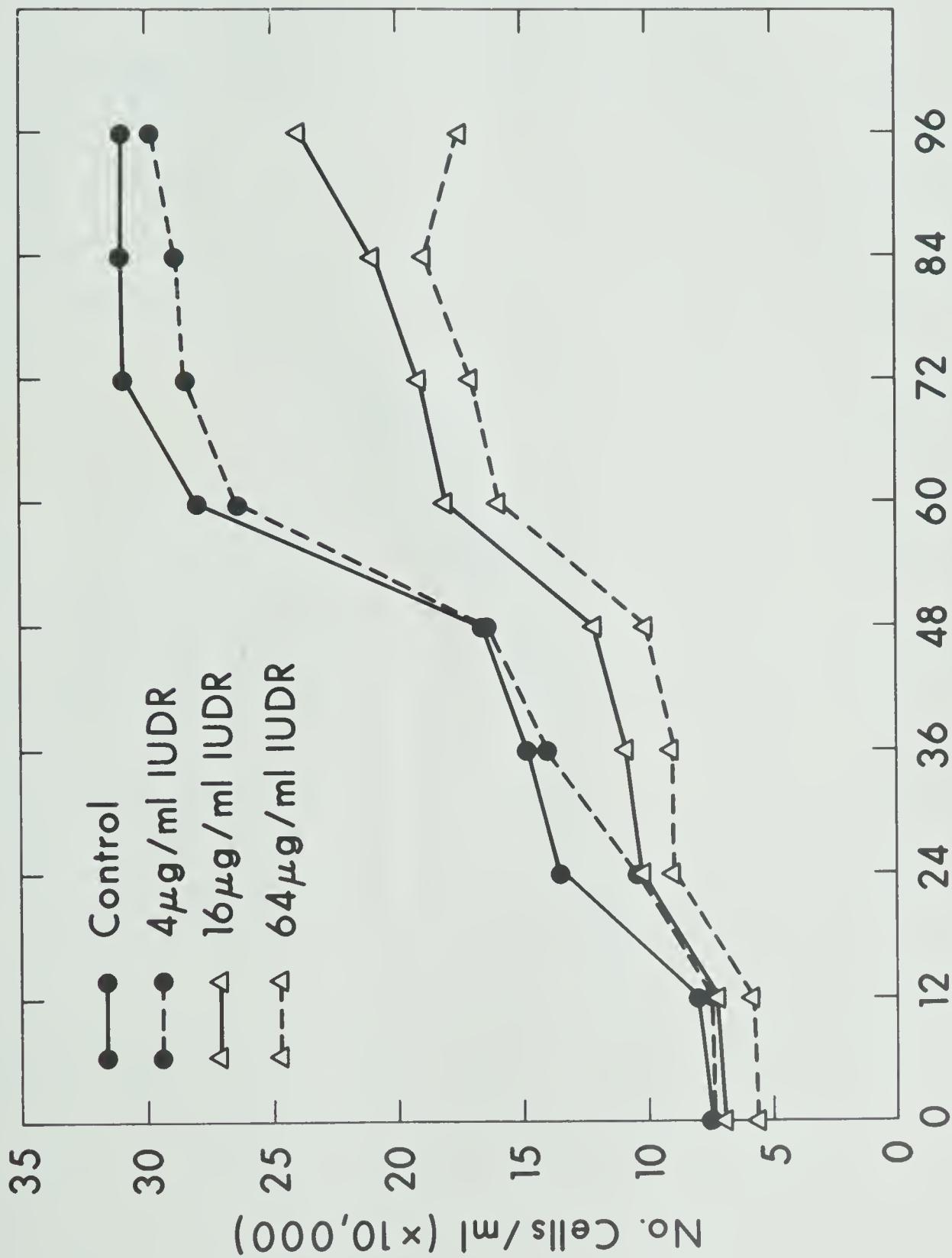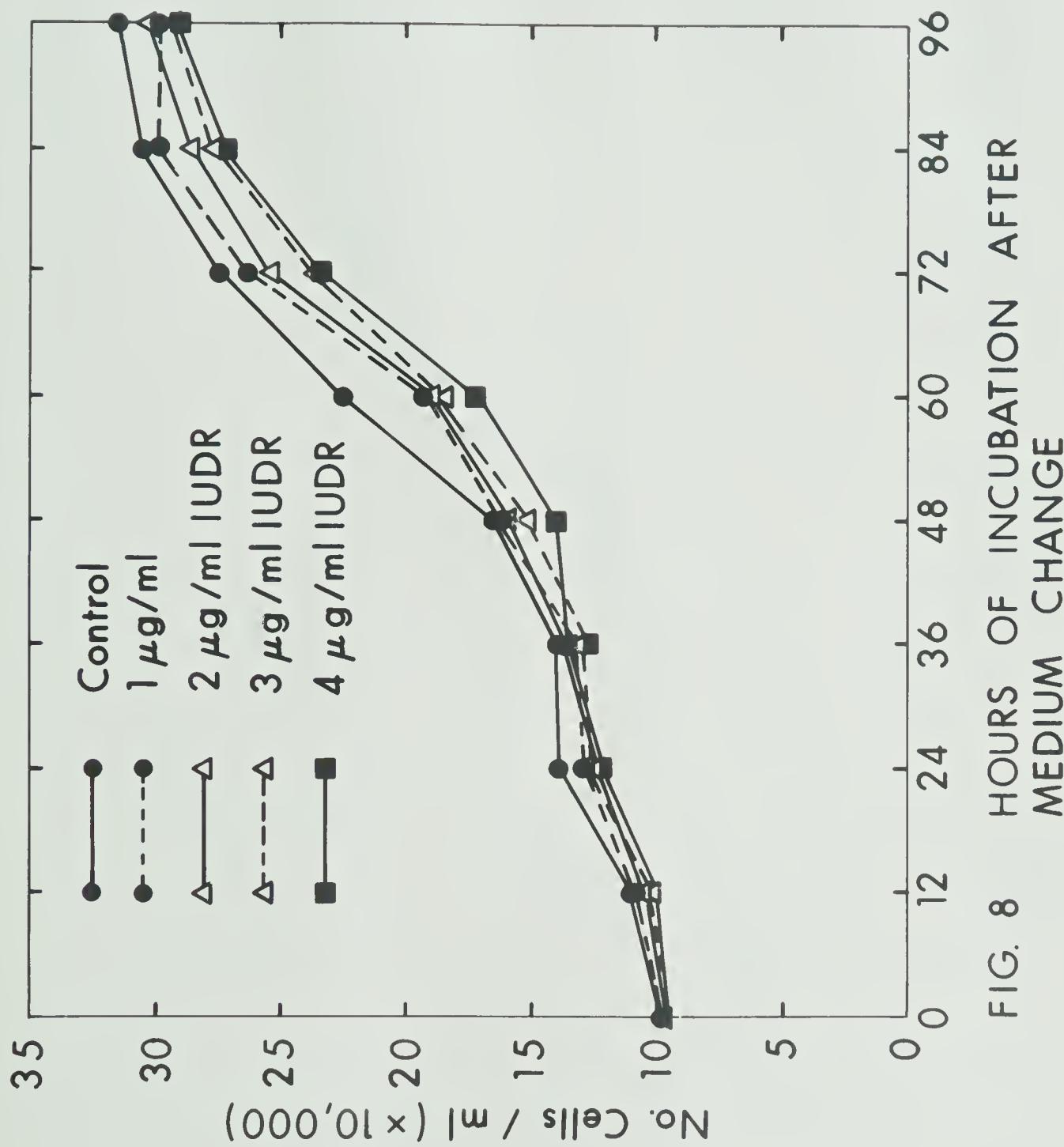
FIG. 8   HOURS OF INCUBATION AFTER
         MEDIUM CHANGE
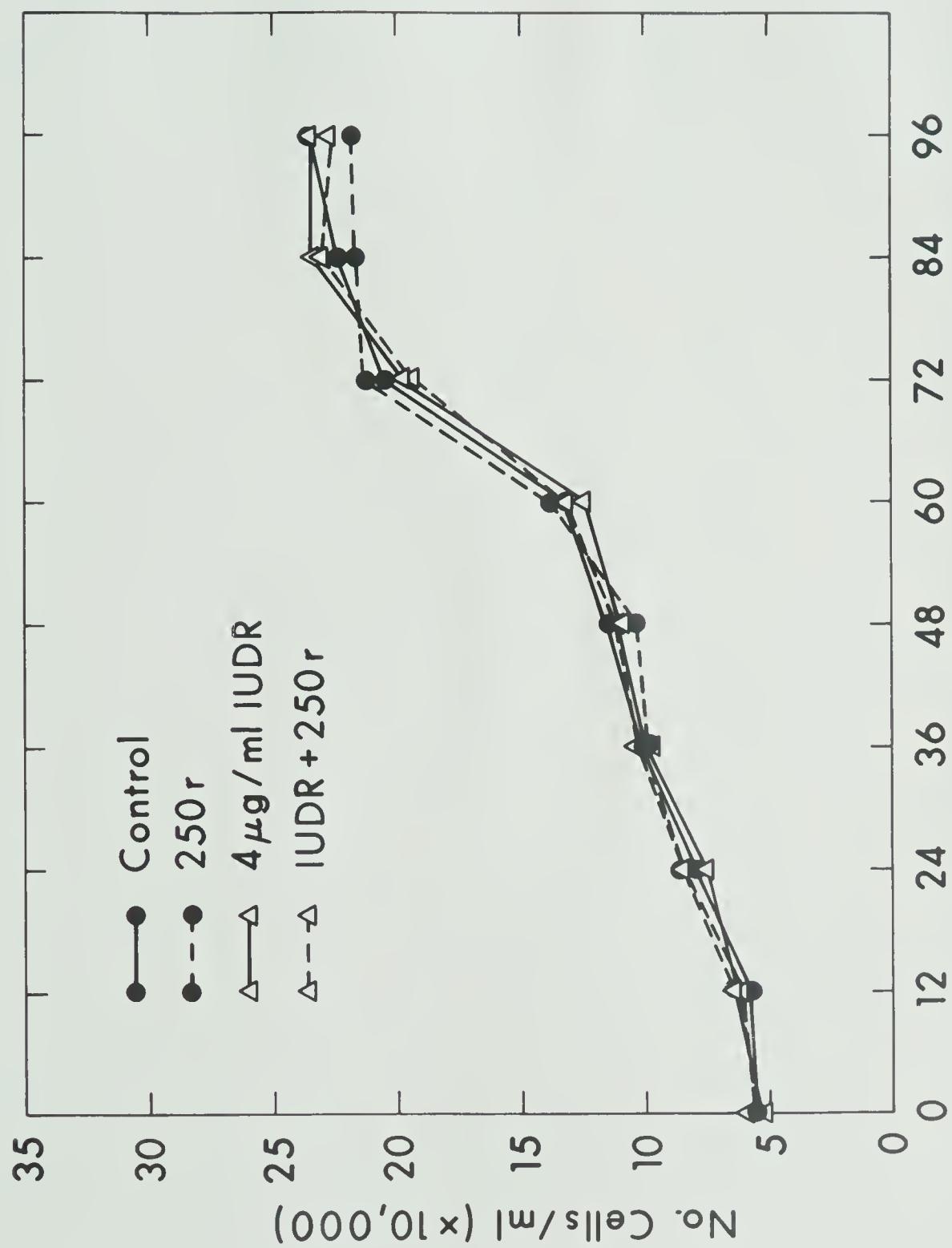
FIG. 9 HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE

FIG. 10(a) HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE

FIG. 10 B

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE

Control
4 μg/ml IUDR
IUDR + 250r
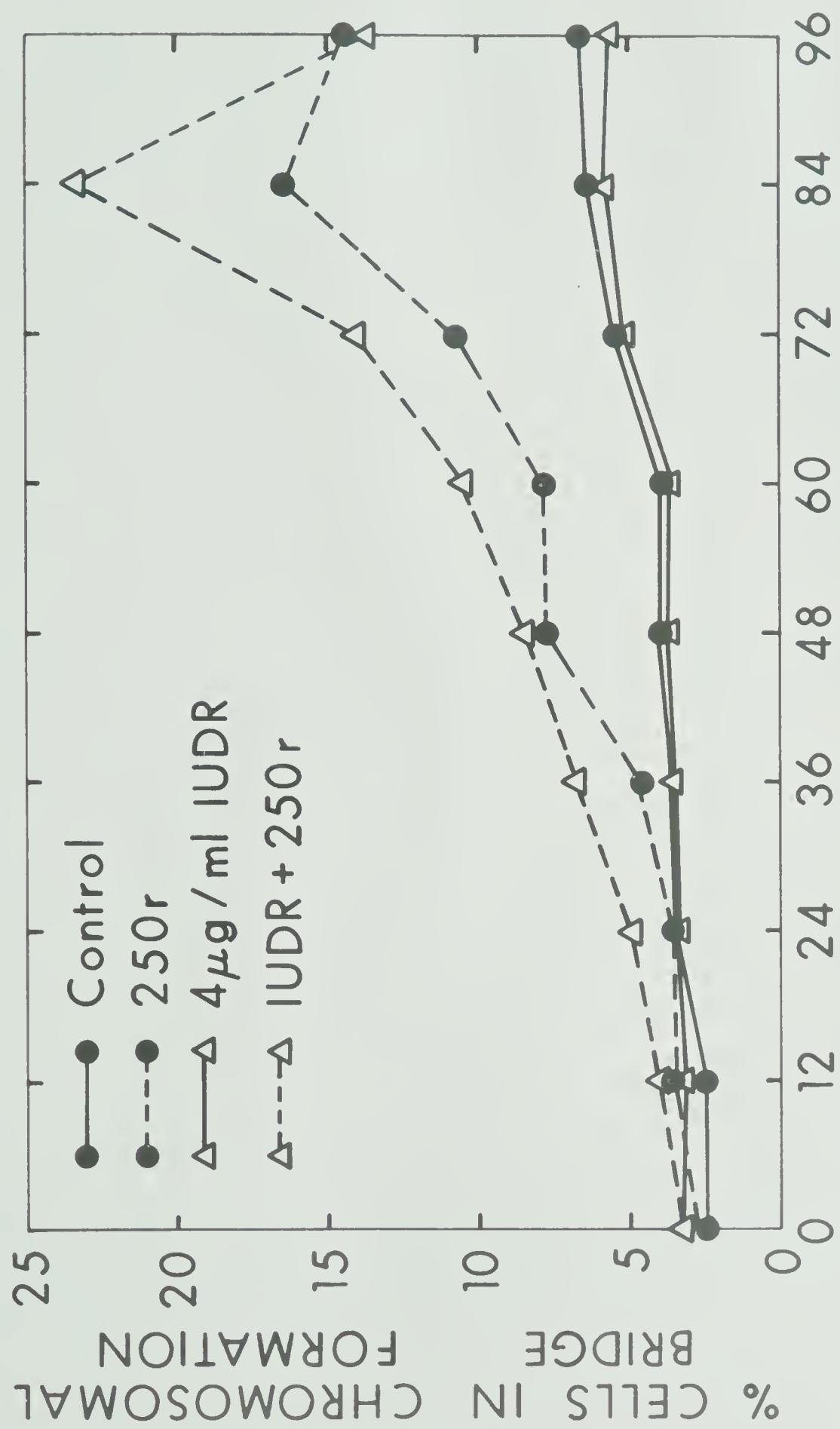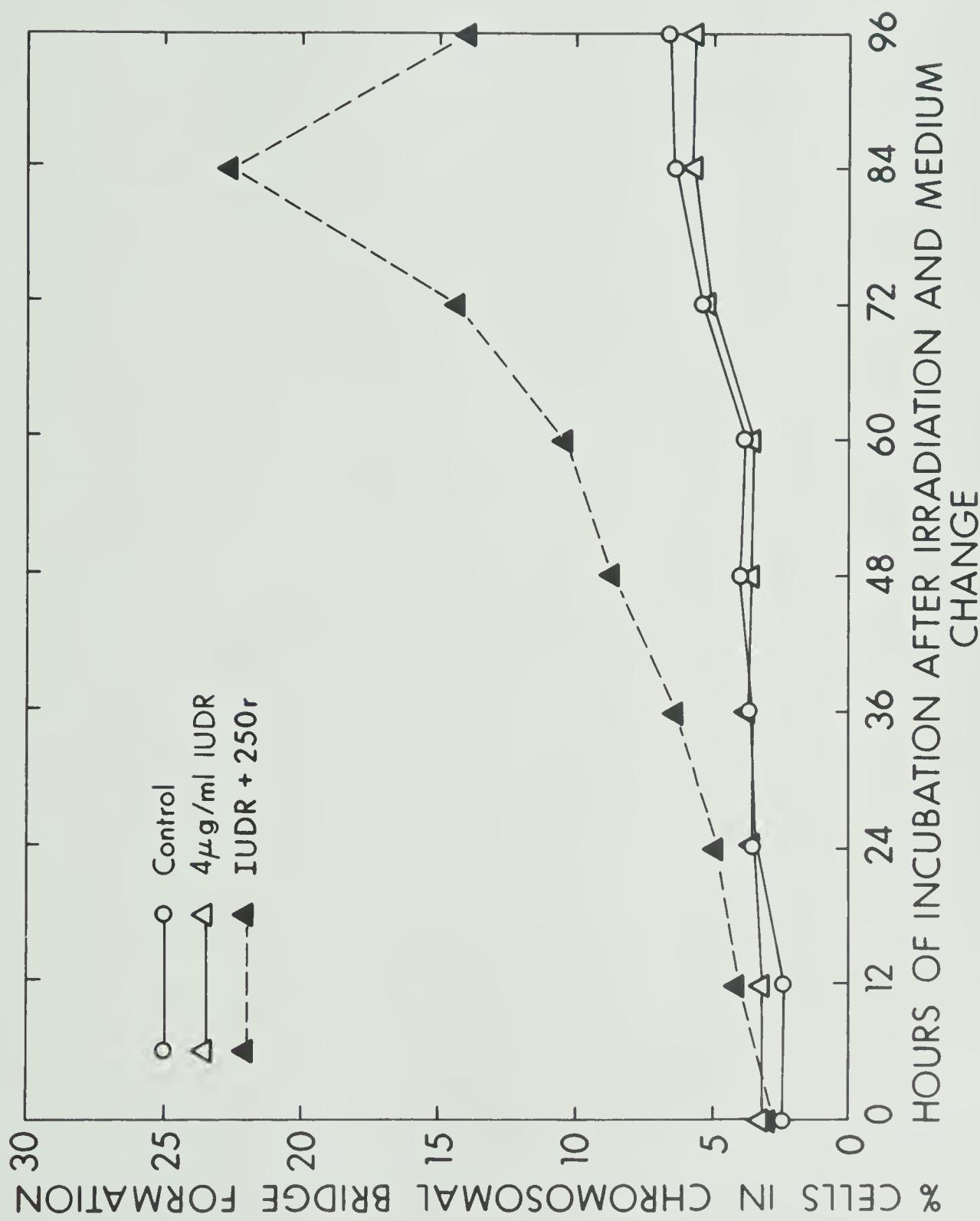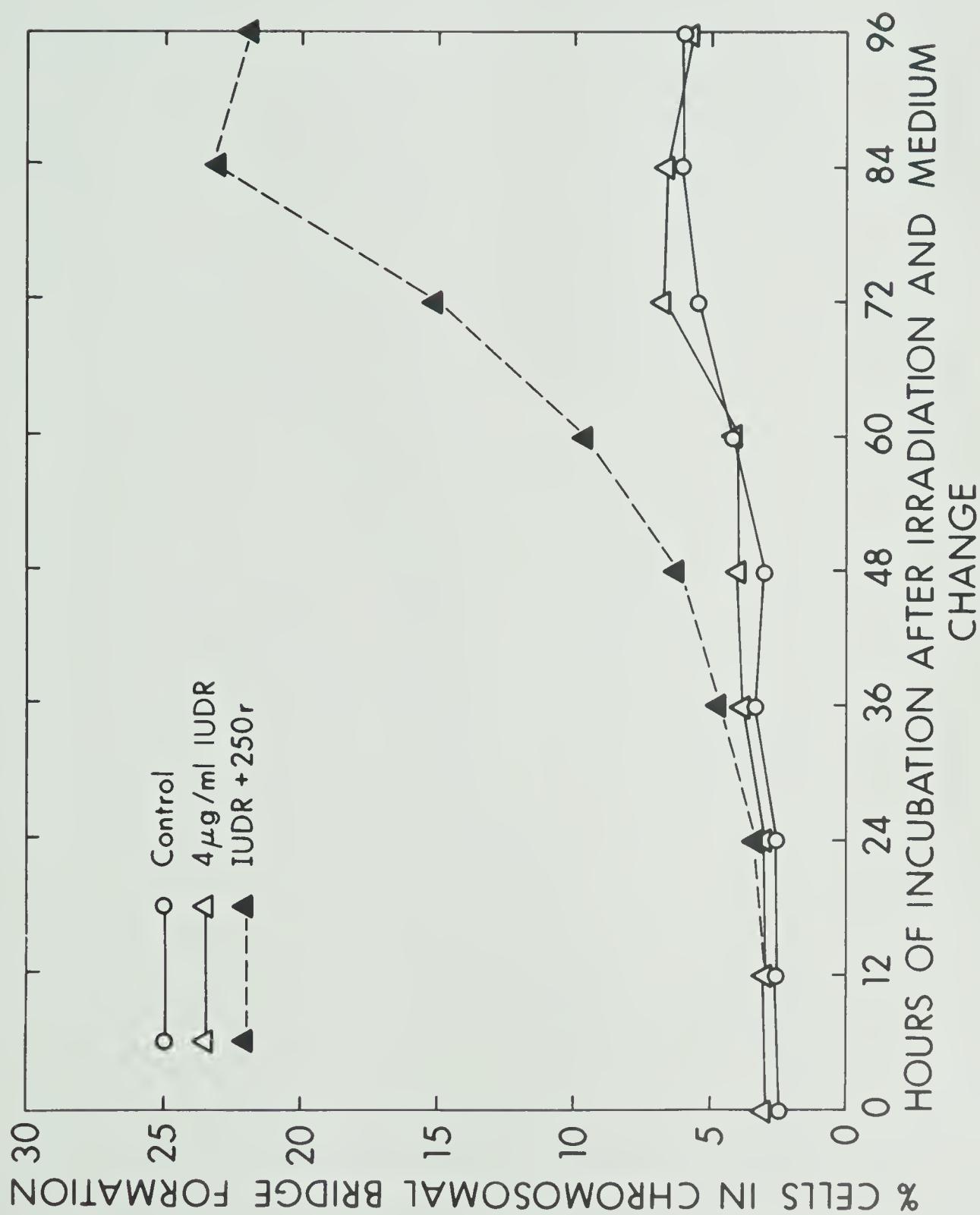
FIG. 11

Fig. 11A. A composite graph of Figs. 9 and 10A demon-
           strating the dependence of chromosomal
           bridge formation on mitotic activity.
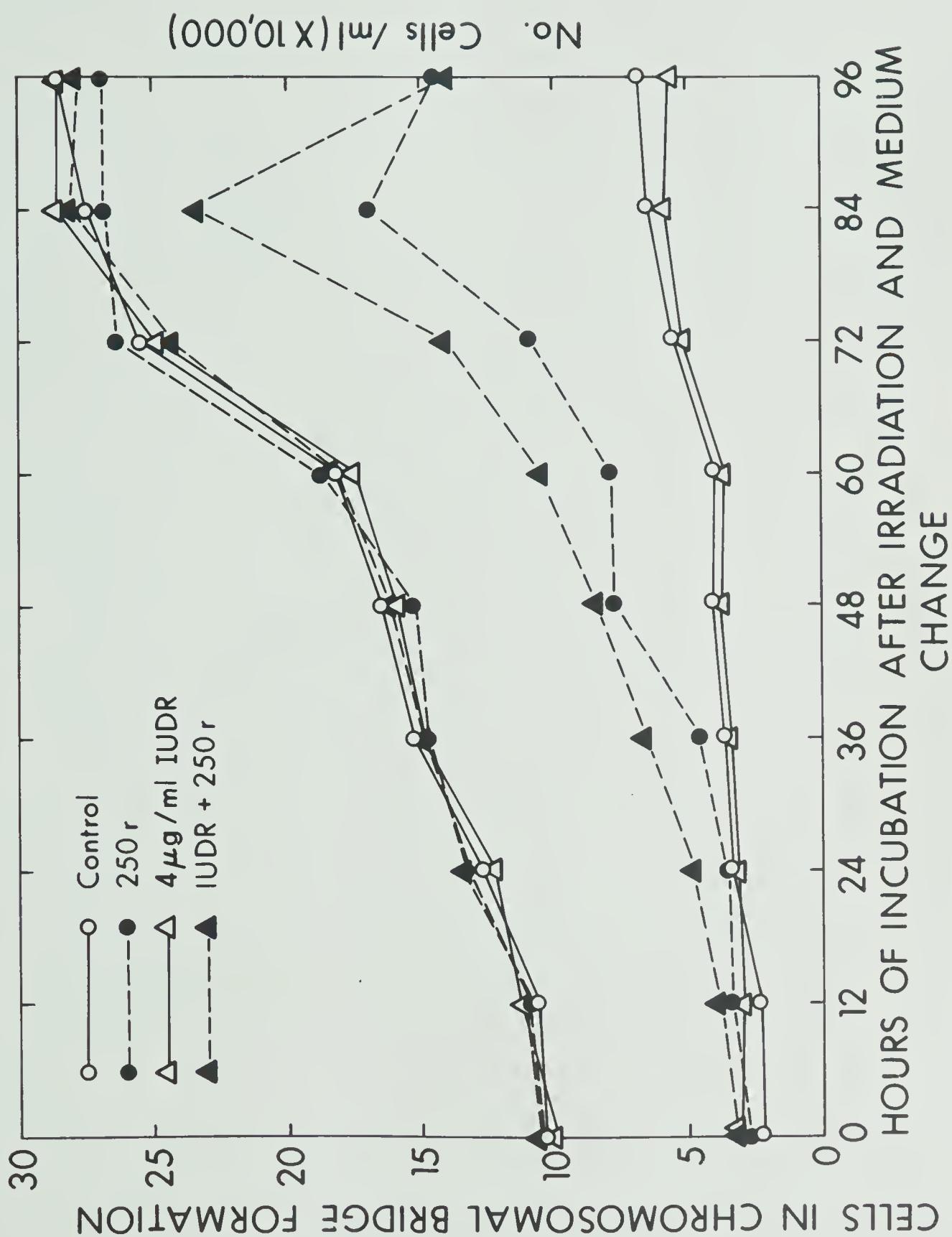
FIG. 11A

Fig. 12.   Two dimensional chromatograph of nucleic acid
            bases.

            A - Thymine,   B - Uracil,   C - Iodouracil

            D - Adenine,   E - Cytosine and F - Guanine

FIG. 13

HOURS OF INCUBATION IN 125-I-UDR
(MEDIUM CHANGED AFTER 5 DAYS)

FIG. 14

STANDARDIZATION CURVE
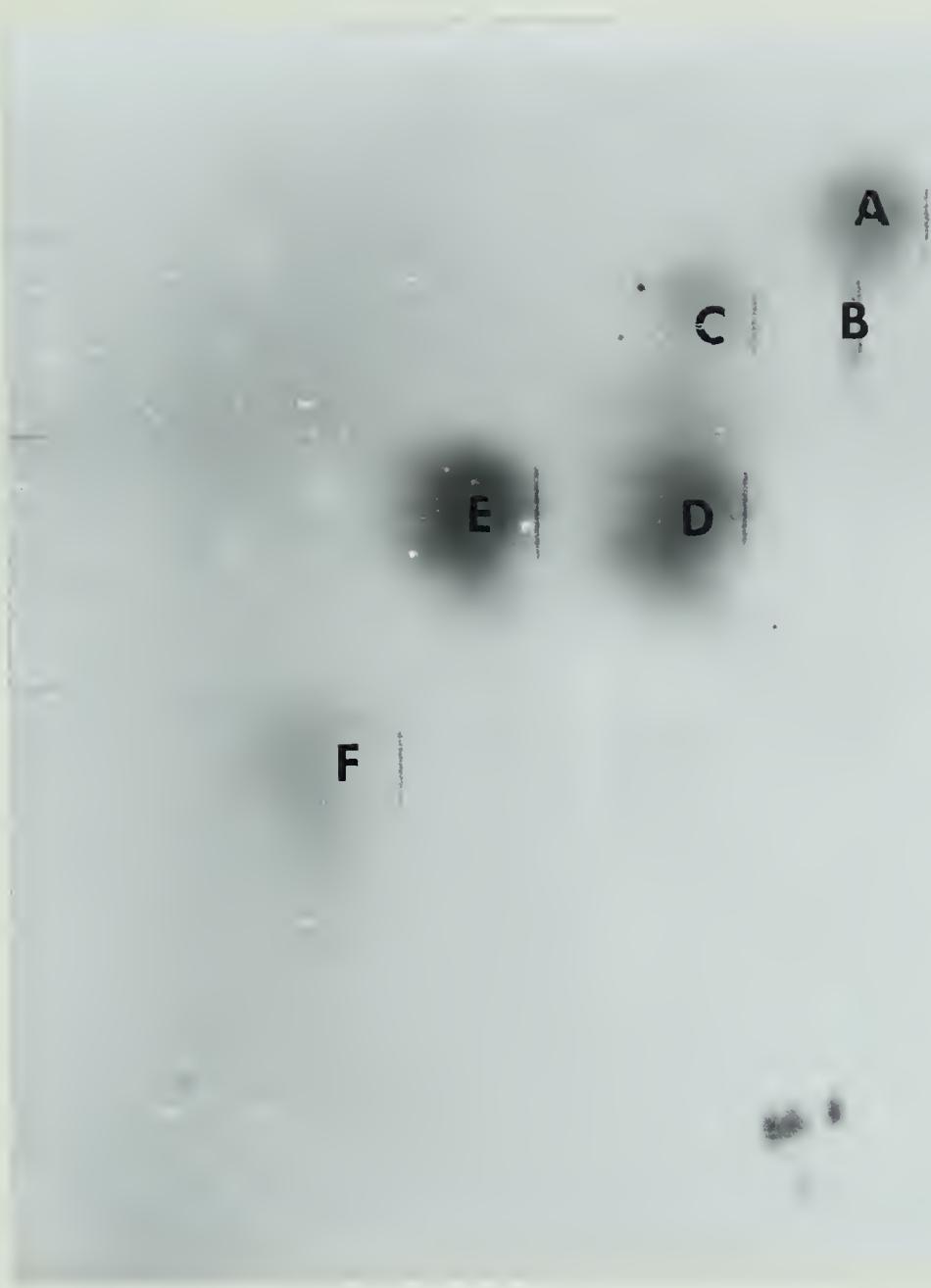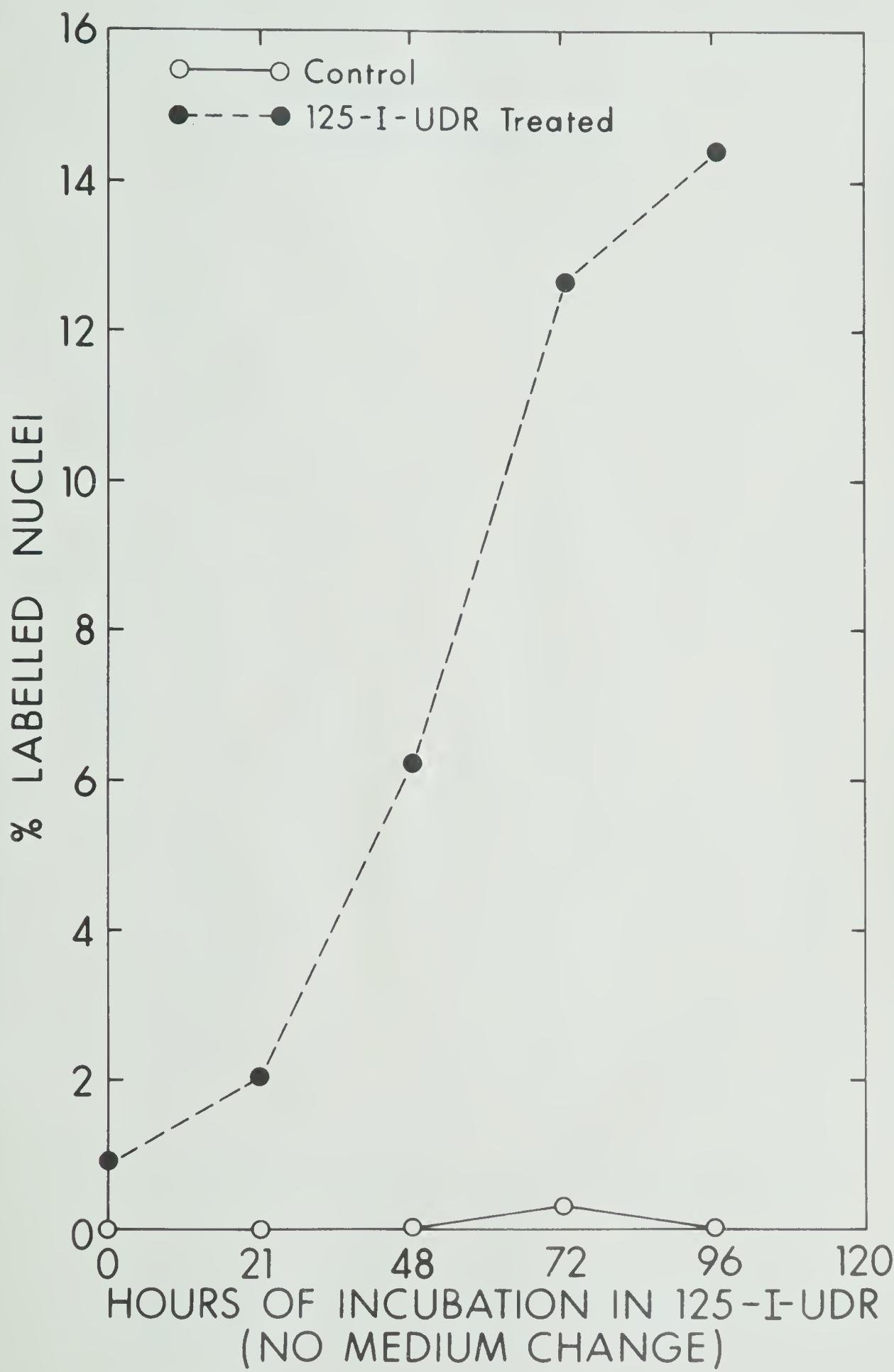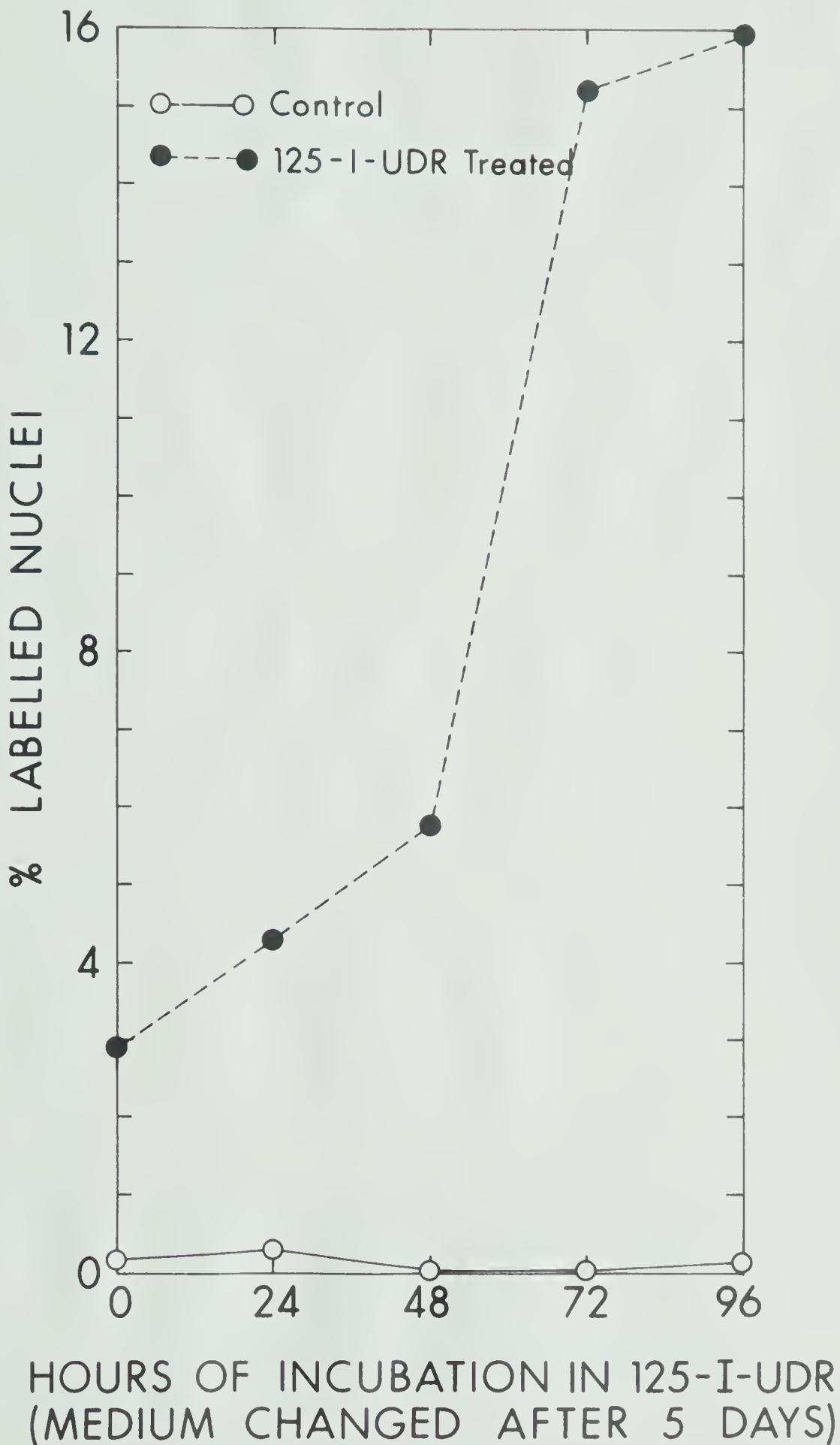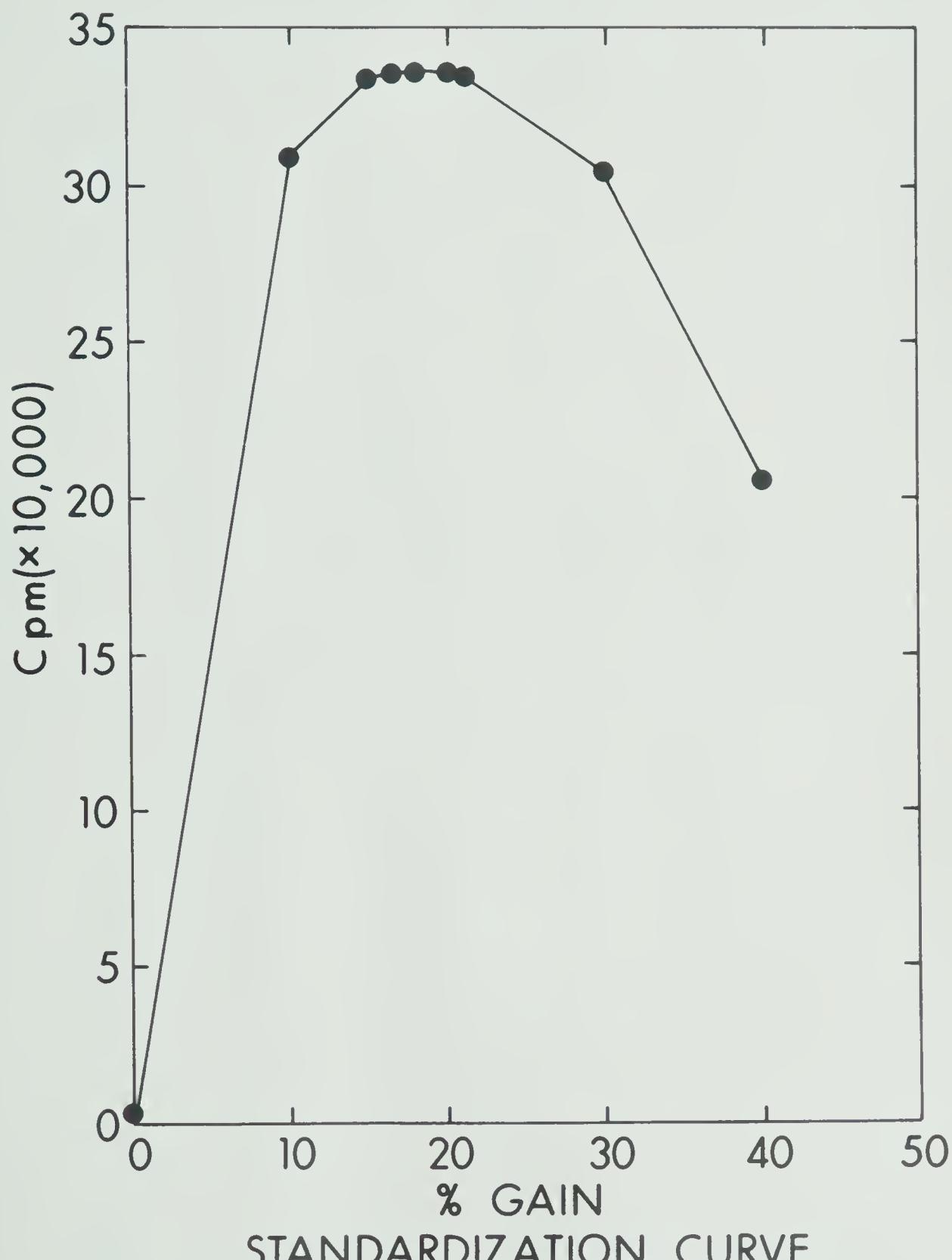
FIG. 15
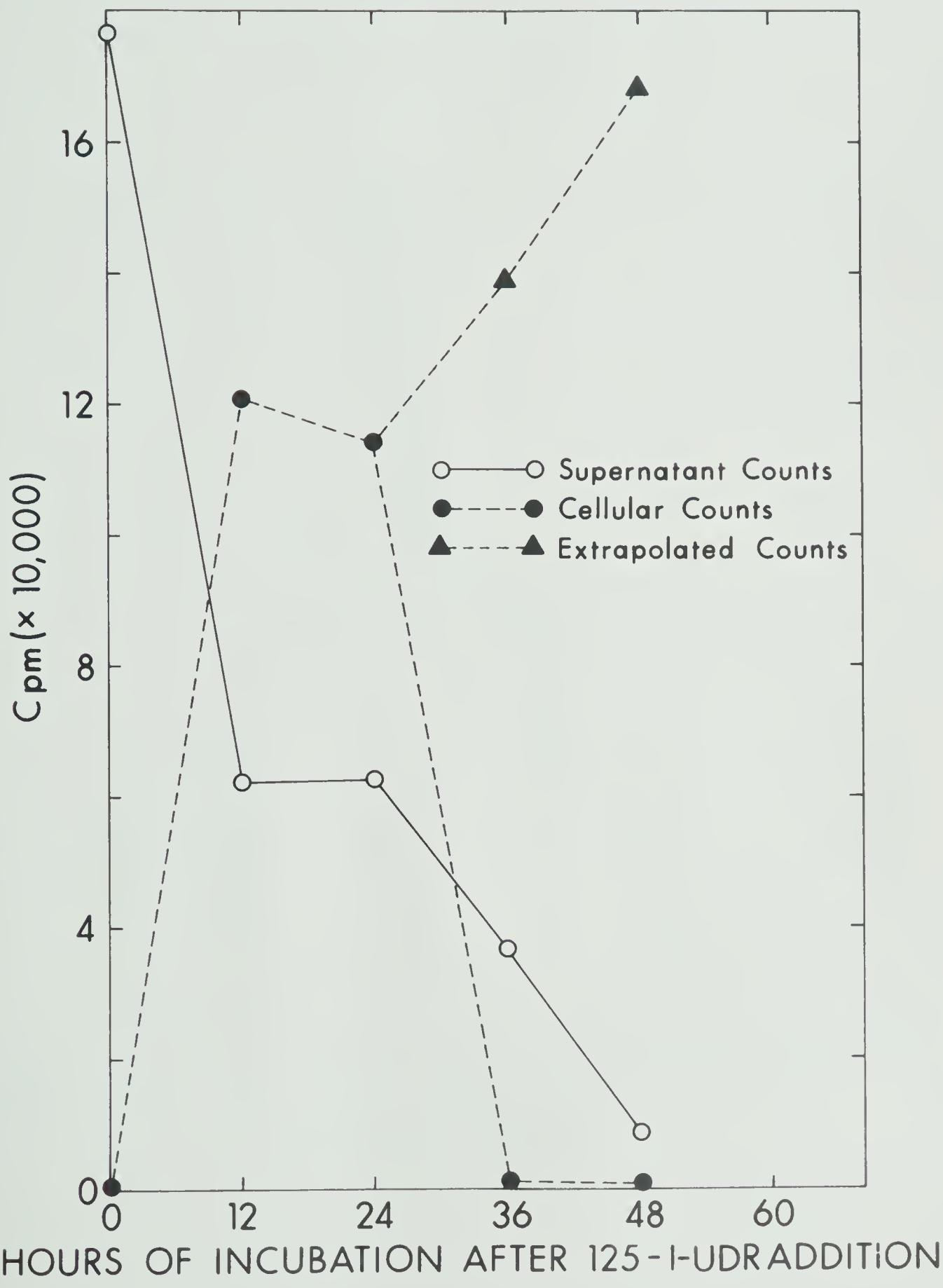
FIG. 16
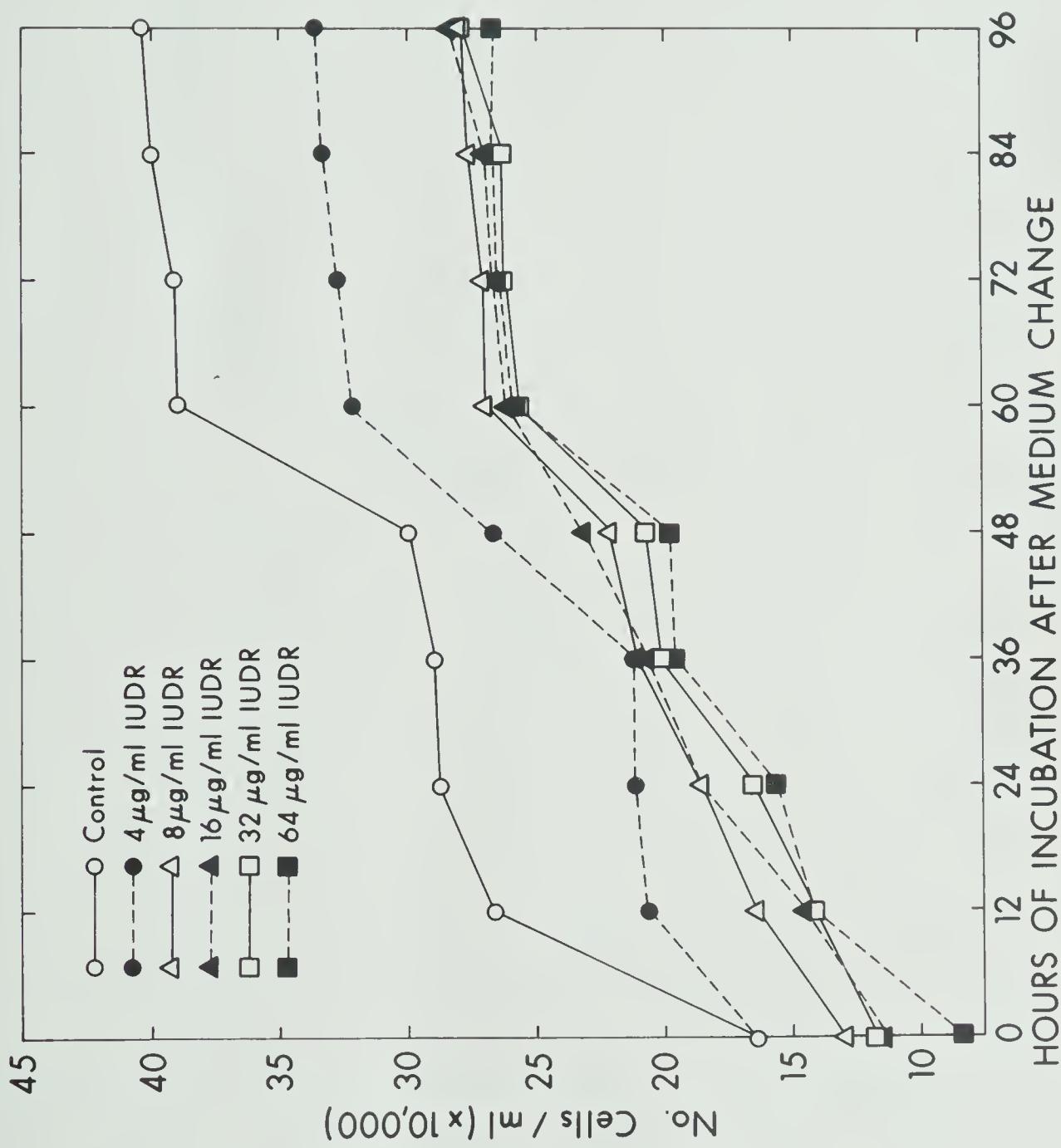
FIG. 17
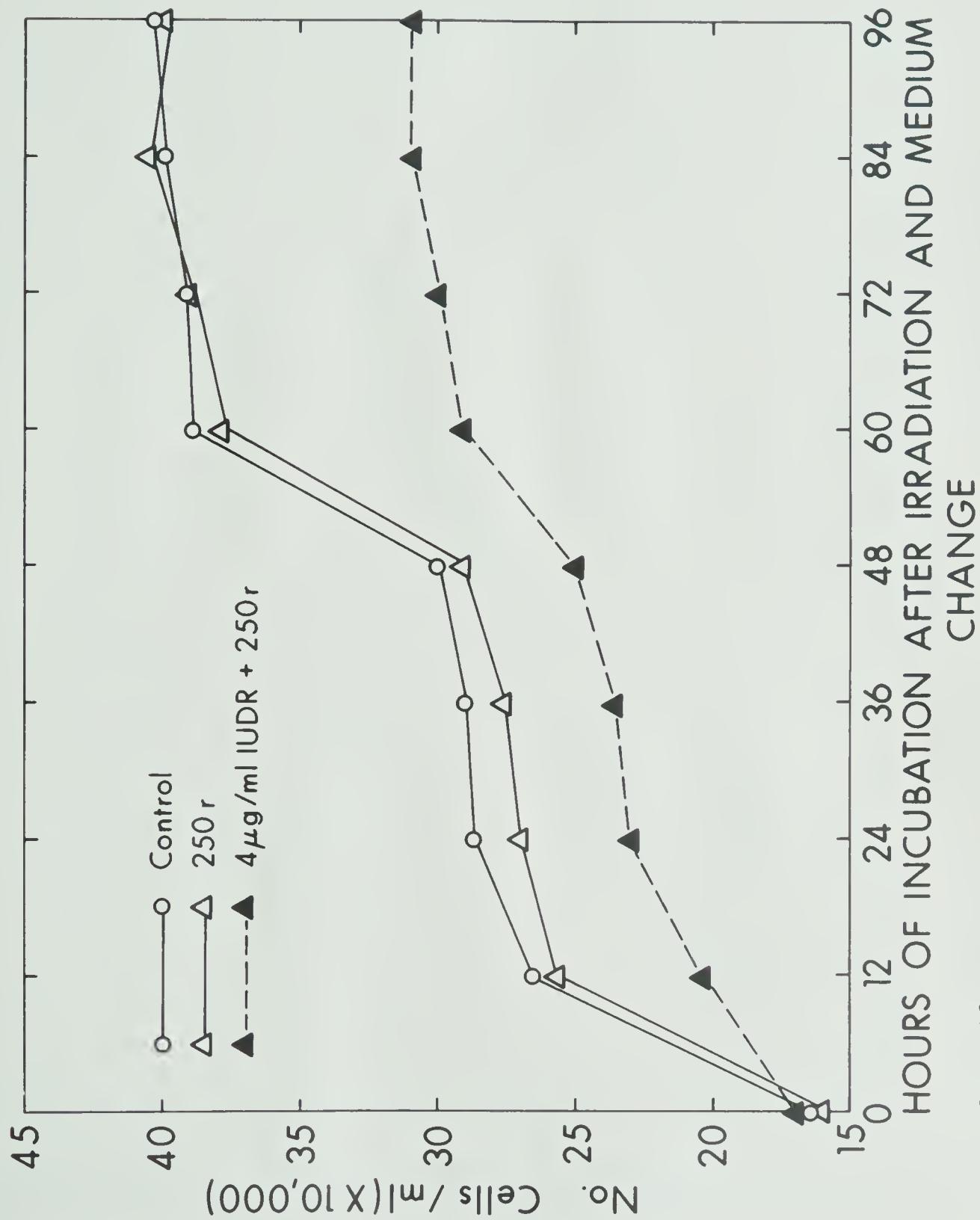
FIG. 18
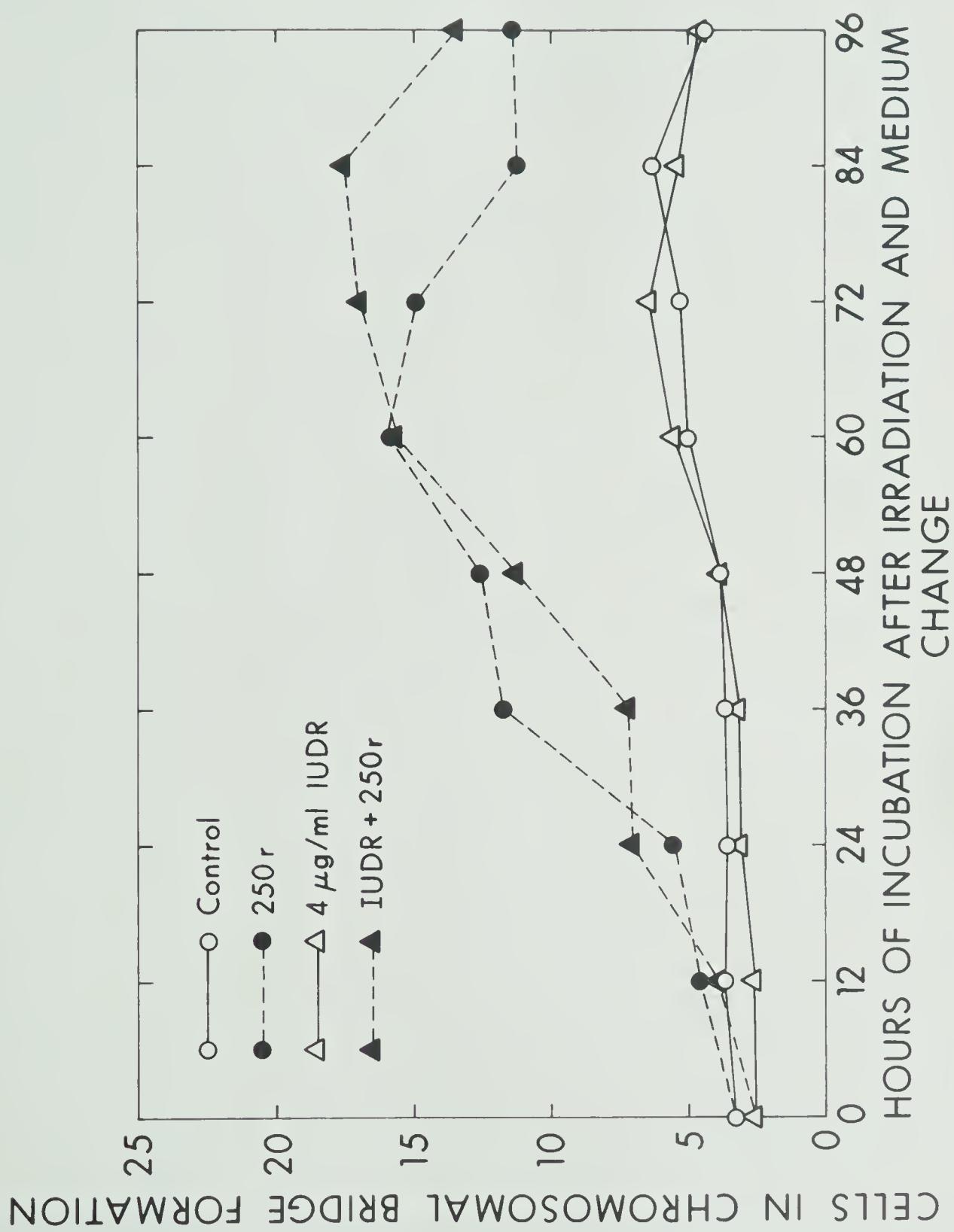
FIG. 20(a)
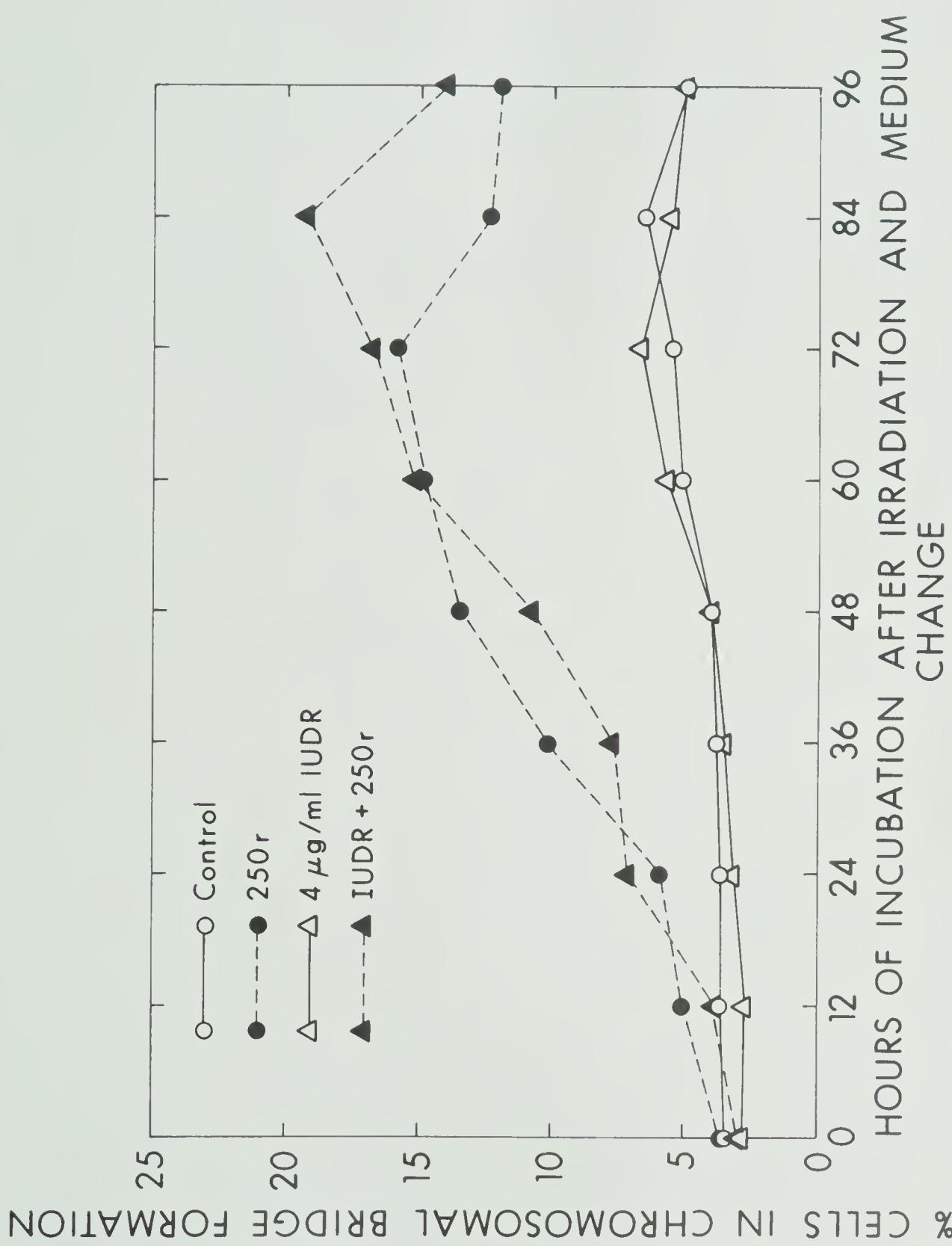
FIG. 20(b)

Fig. 21.   A composite graph of Figs. 17, 18 and 19 demonstrating the dependence of chromosomal bridge formation on mitotic activity.

FIG. 21

FIG. 22

FIG. 23

HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM CHANGE

No. Cells/ml (×10,000)

○─○ Control
△─△ 64 μg/ml ICDR
□─□ 64 μg/ml ICDR + 250
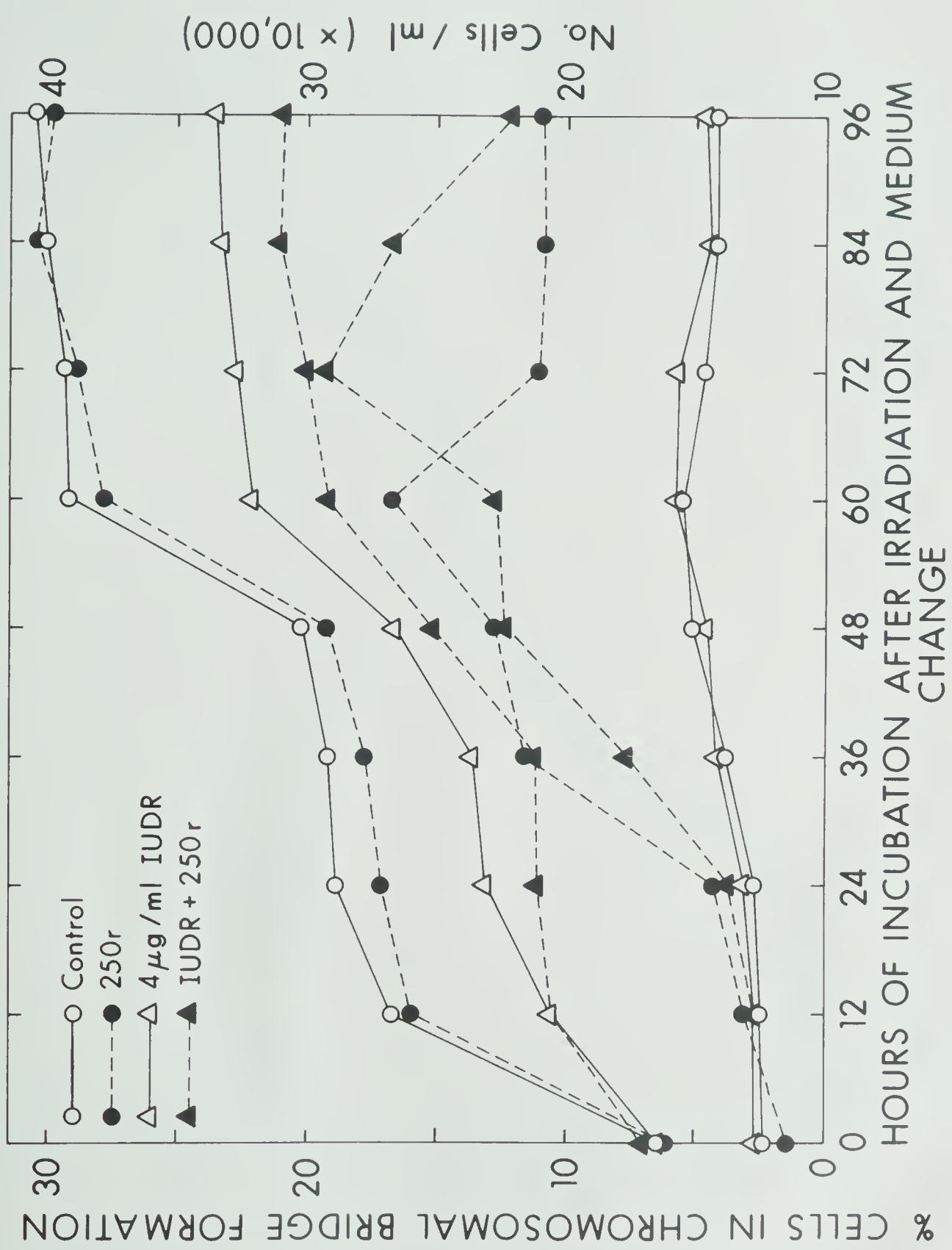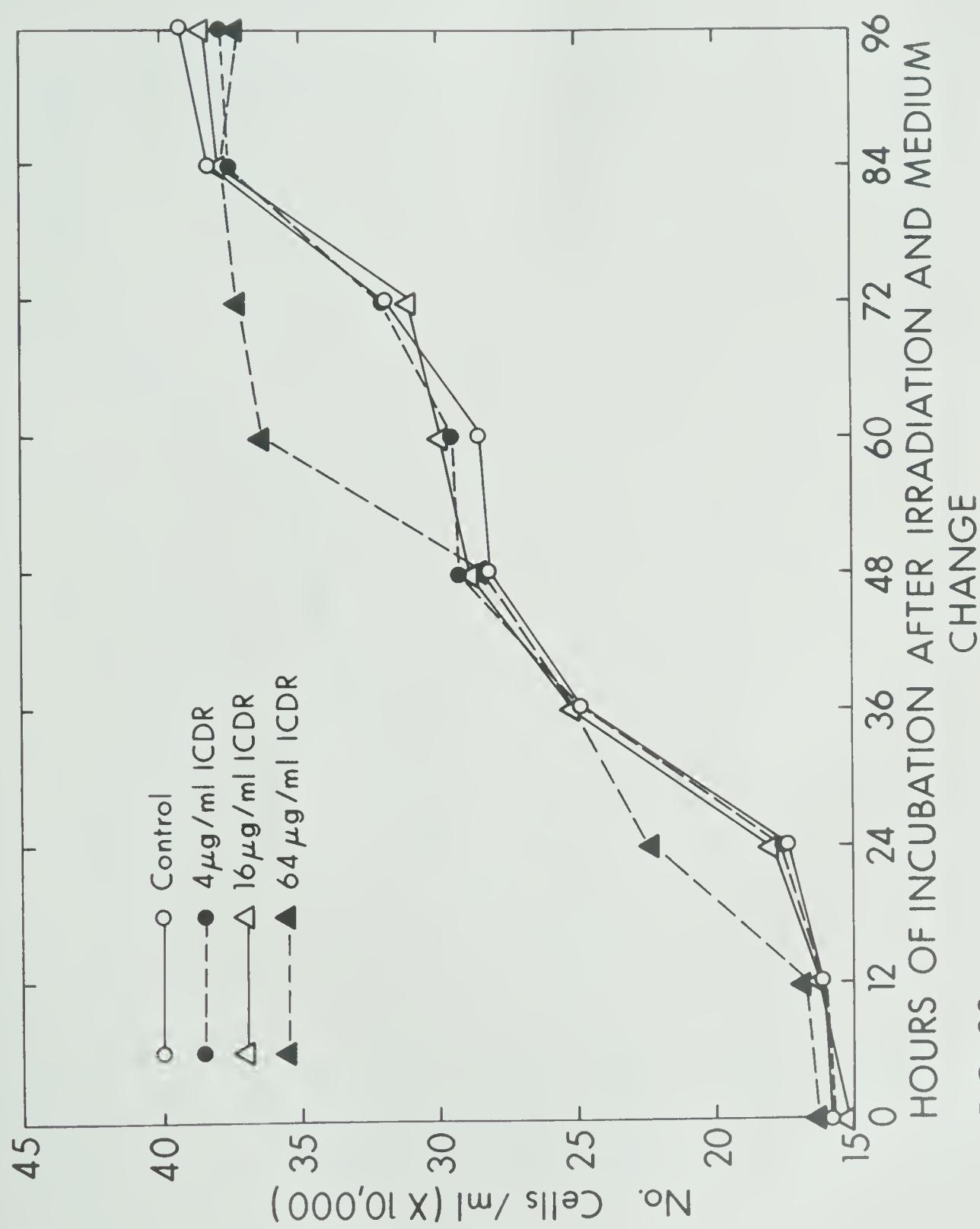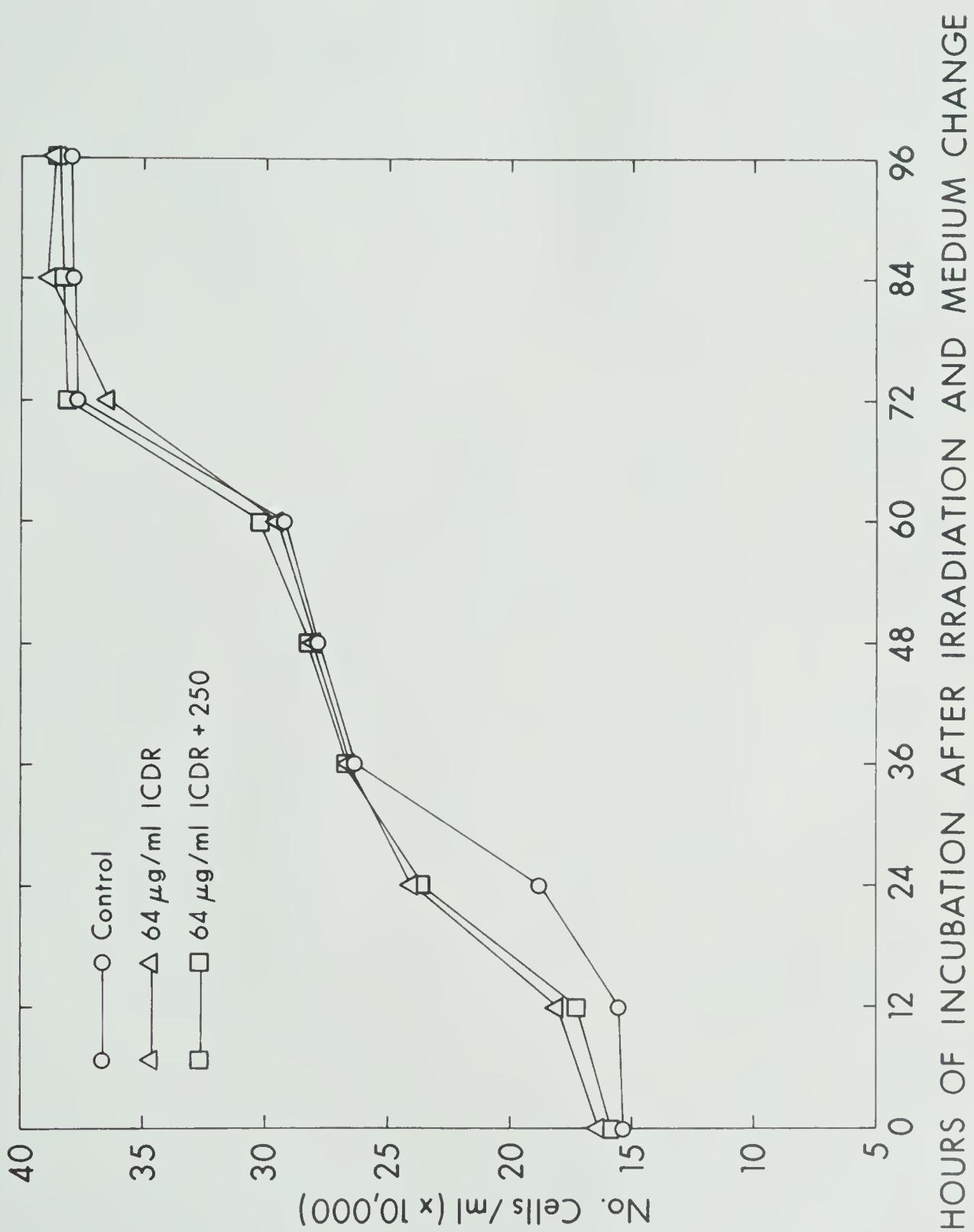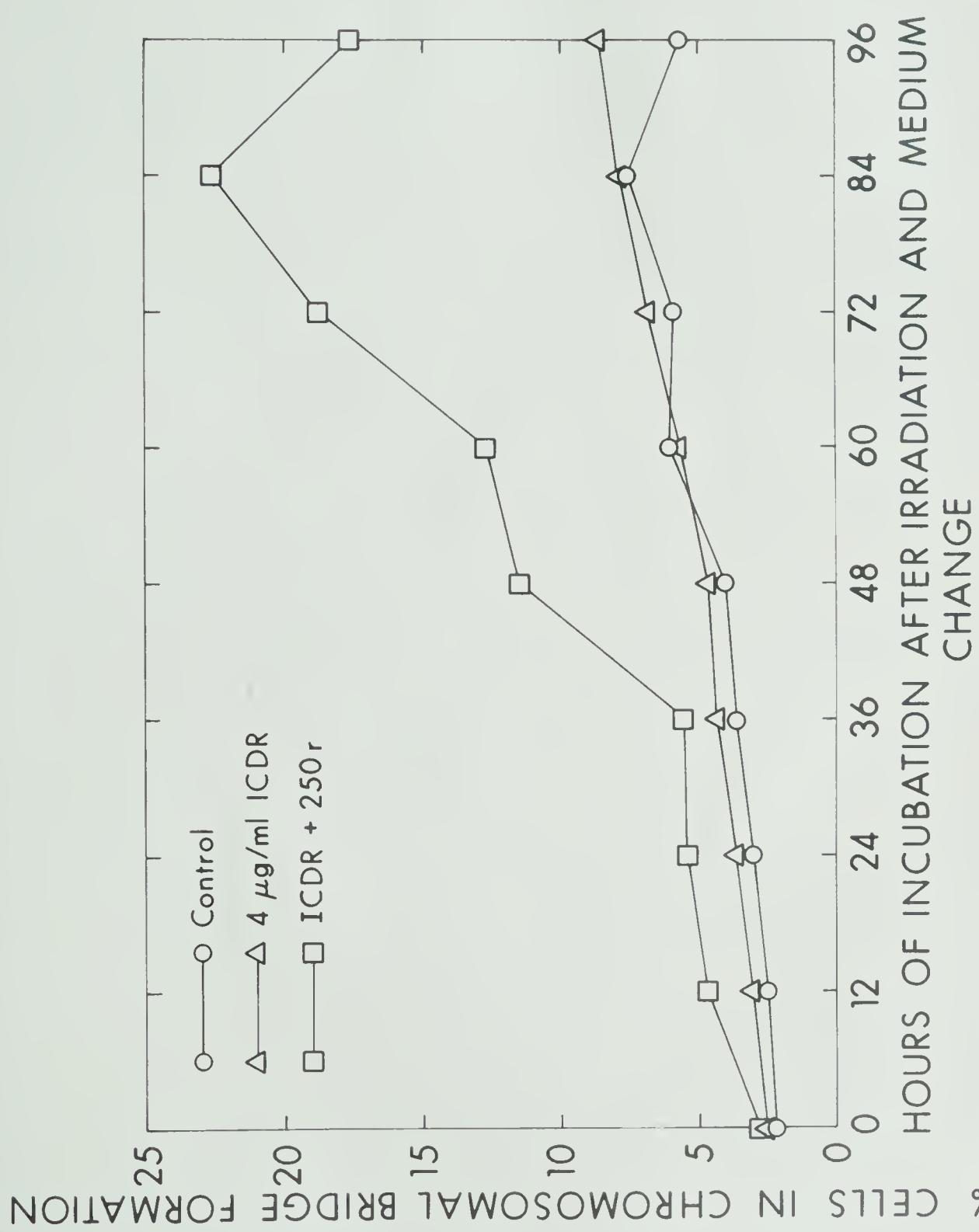
FIG. 24

Fig. 24A. A composite graph of Figs. 22 and 24 demon-
strating the dependence of chromosomal
bridge formation on mitotic activity.
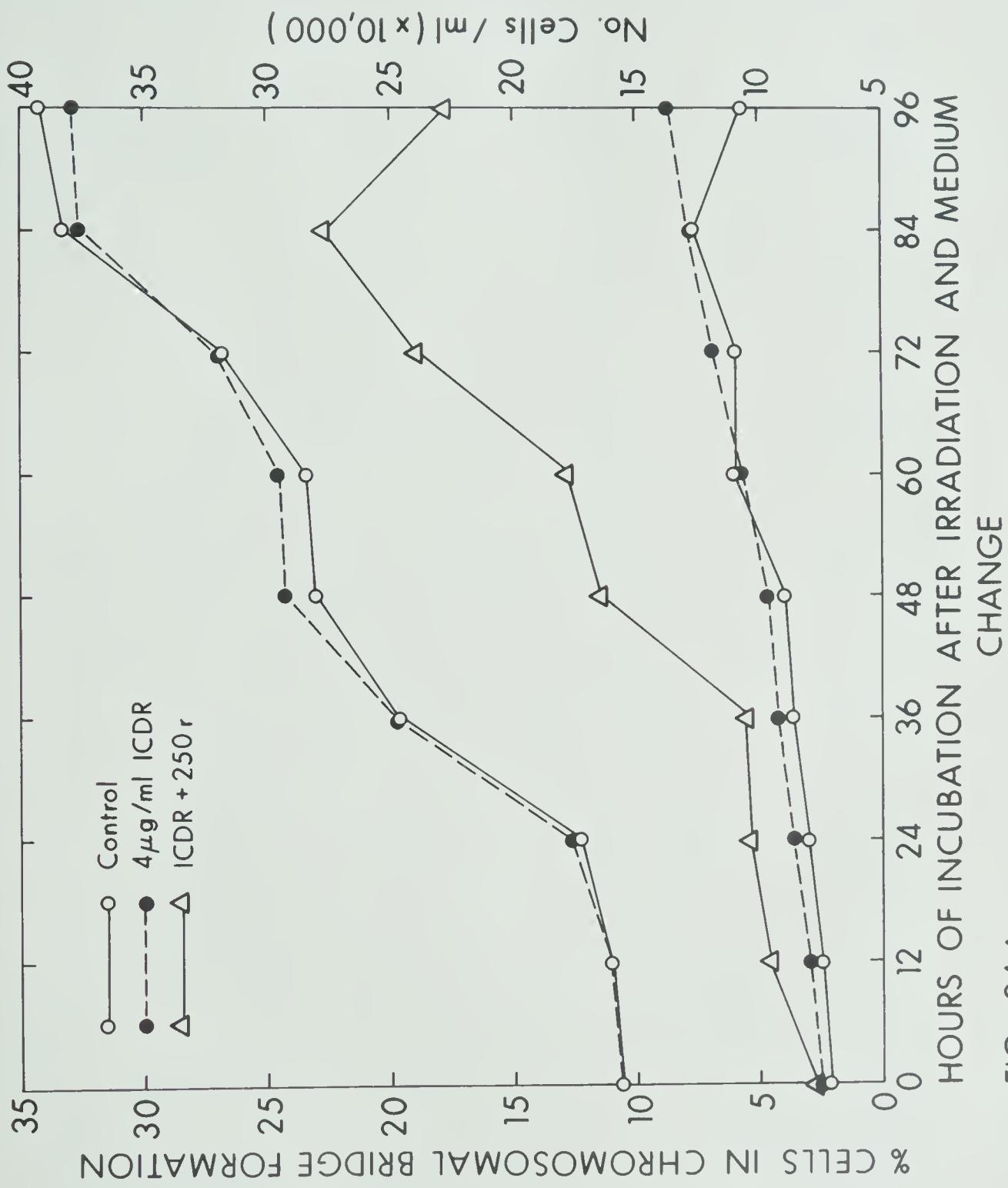
FIG. 24 A
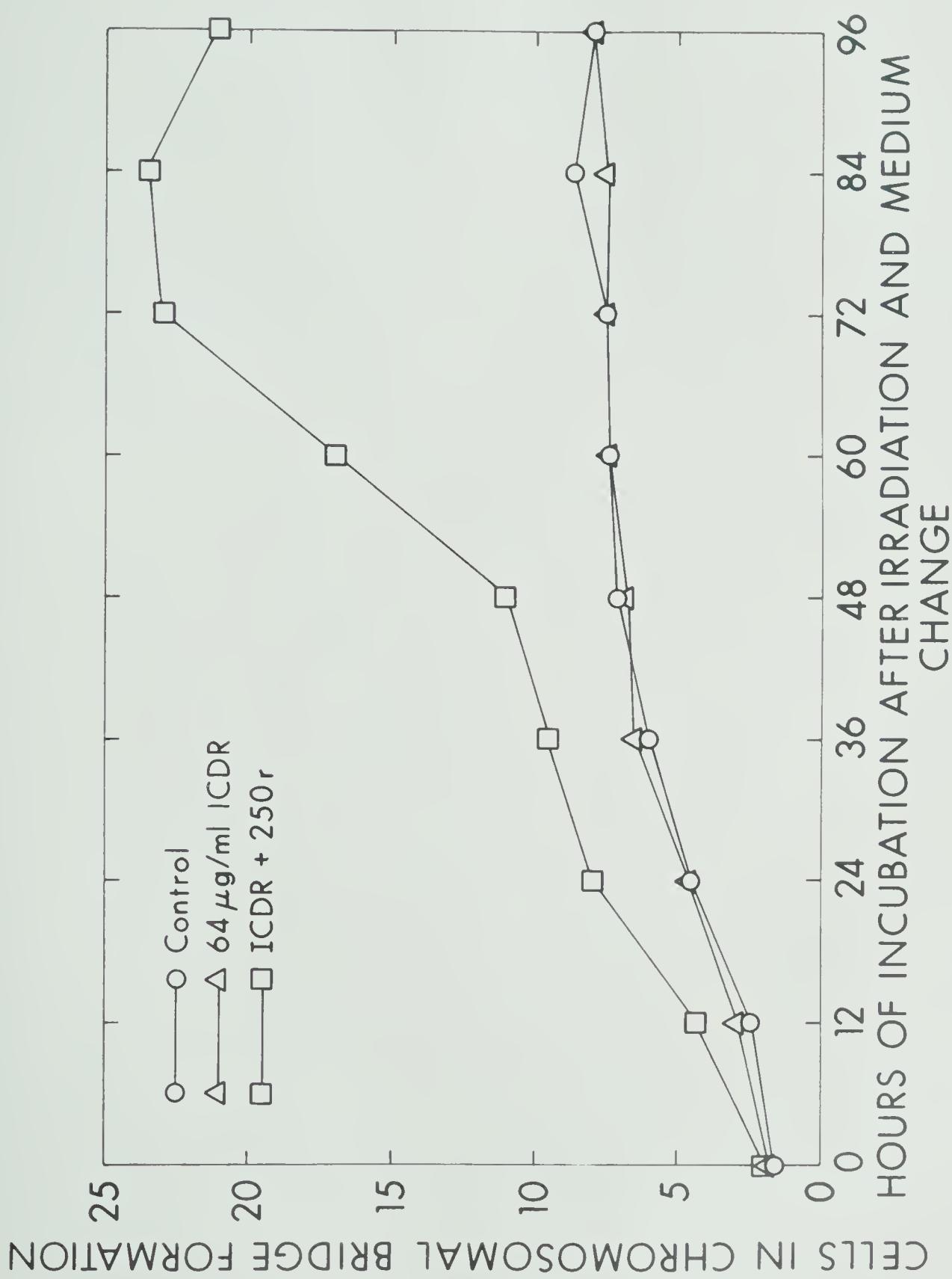
FIG. 25

Fig. 25A.   A composite graph of Figs. 23 and 25 demon-
            strating the dependence of chromosomal
            bridge formation on mitotic activity.

No. Cells/ml (X10,000)

HOURS OF INCUBATION AFTER IRRADIATION AND MEDIUM
CHANGE

FIG. 25 A

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

Control
64 μg/ml ICDR
ICDR + 250r
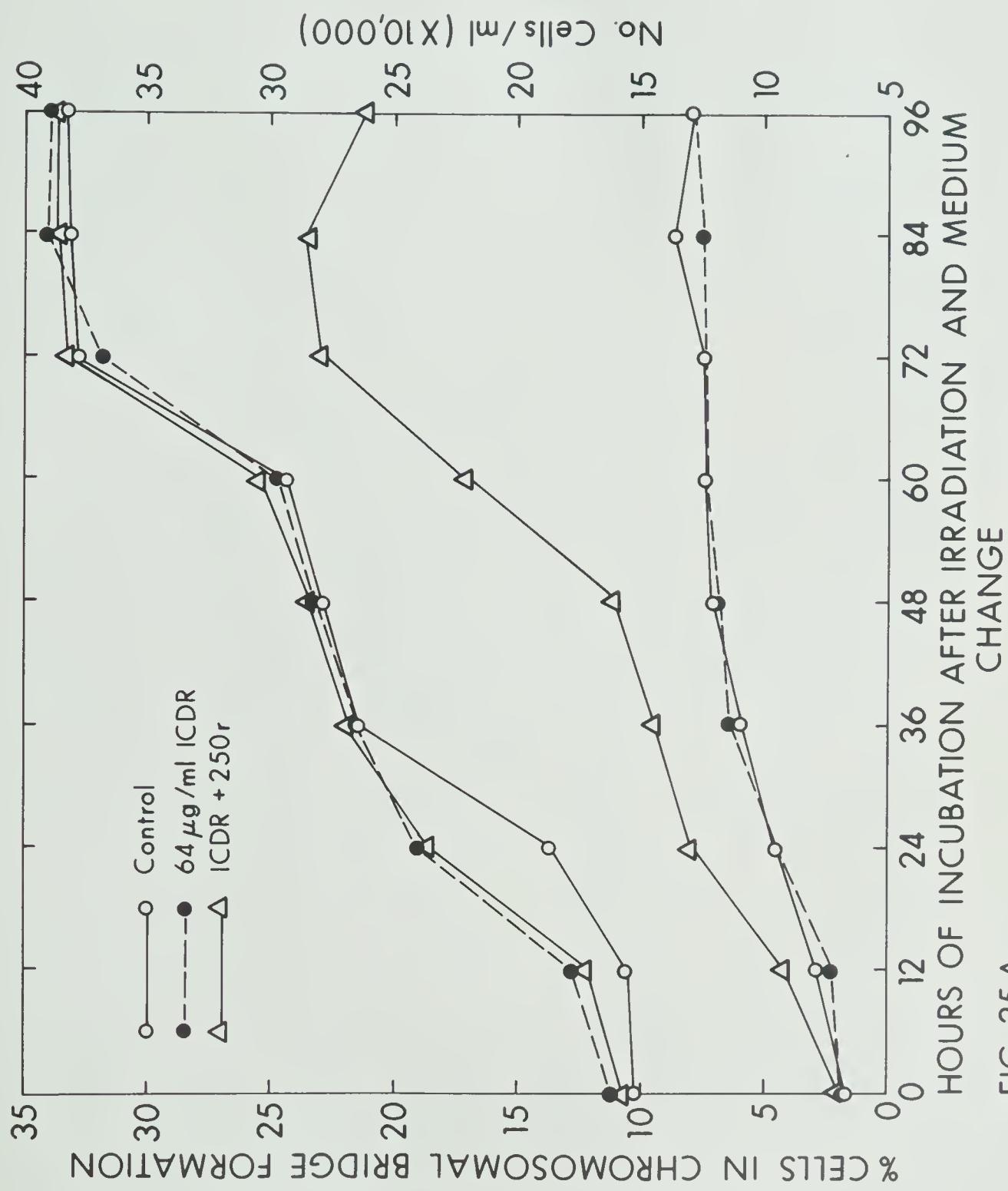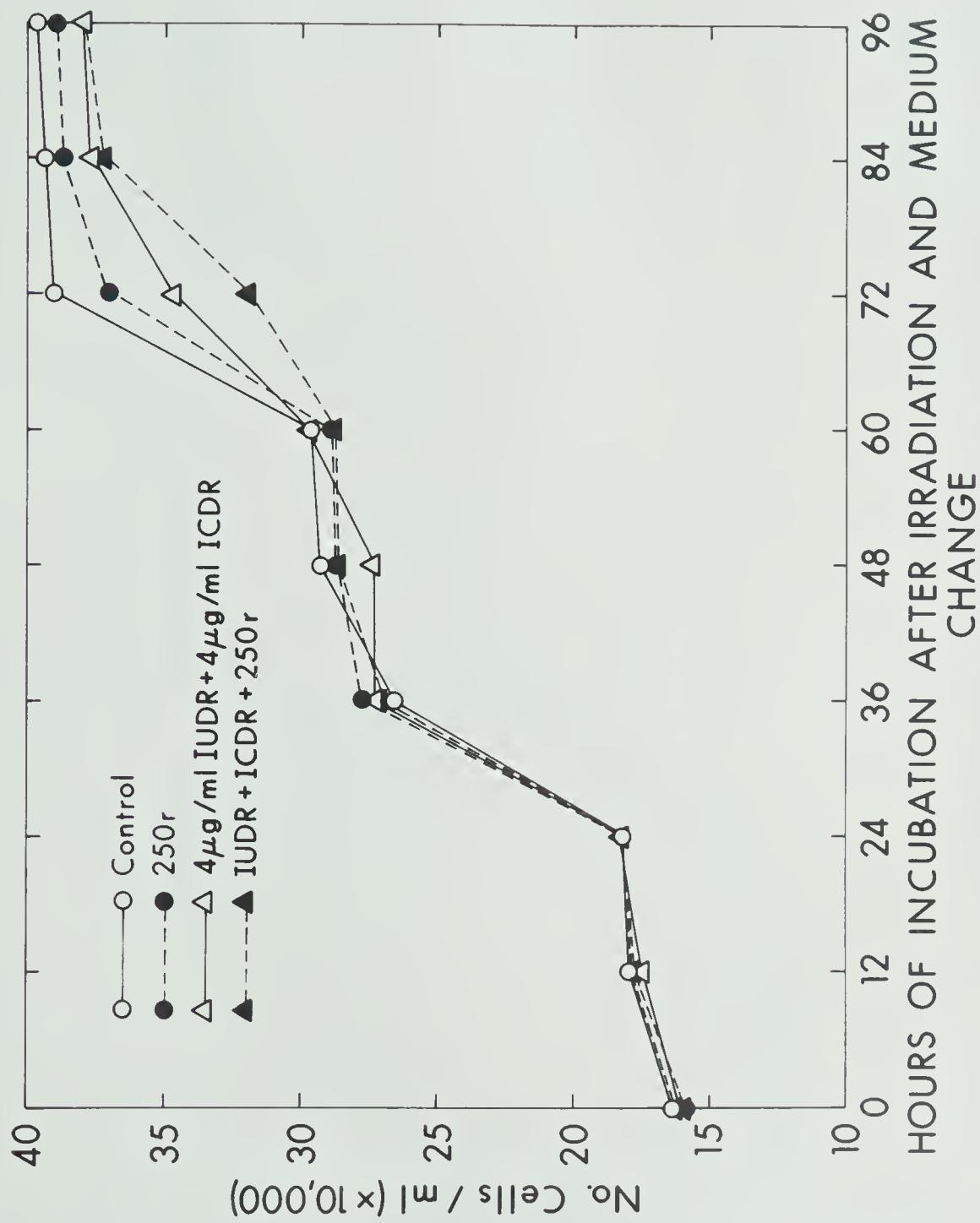
FIG. 26
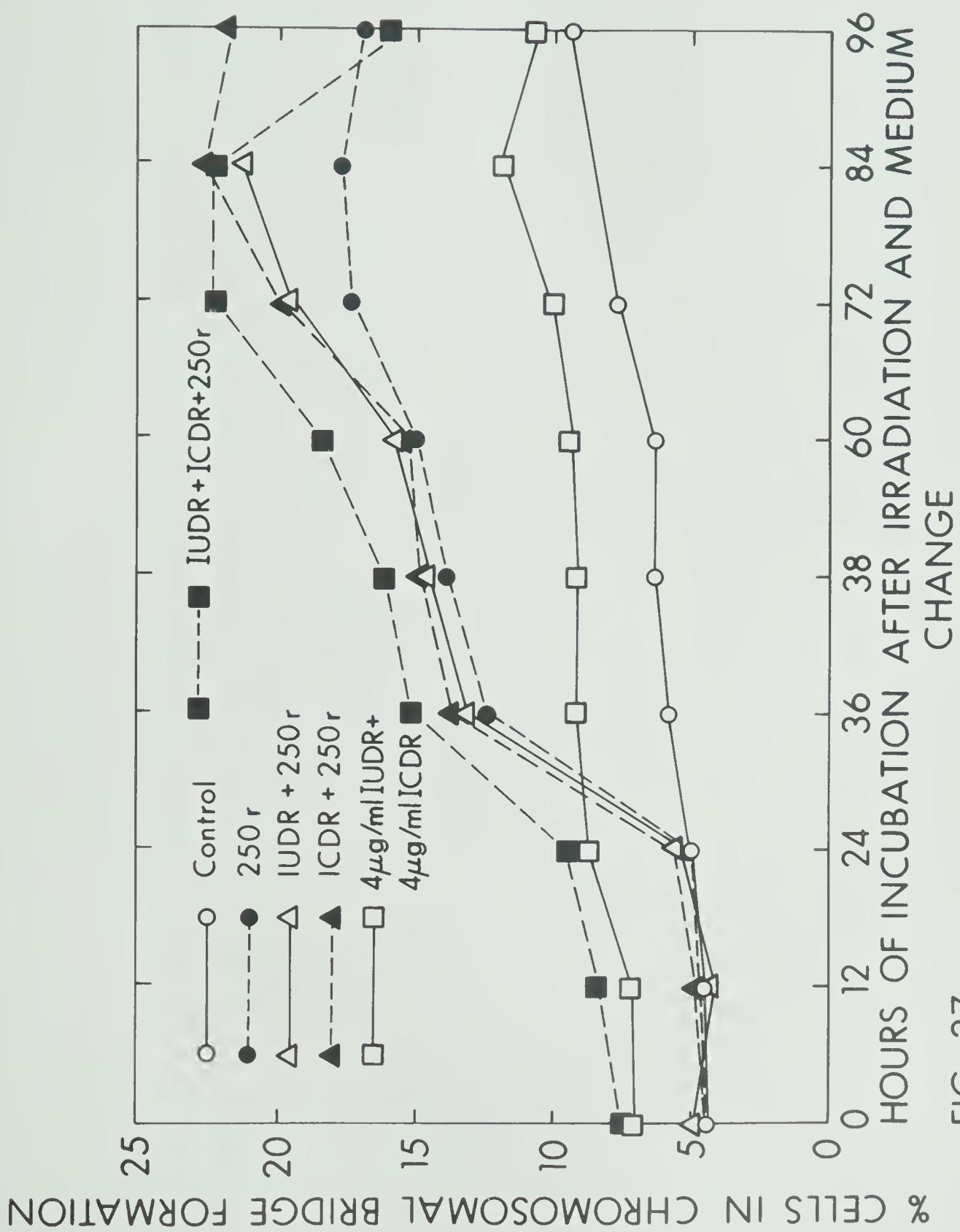
FIG. 27

Fig. 27A. A composite graph of Figs. 26 and 27 demon-
            strating the dependence of chromosomal
   bridge formation on mitotic activity.
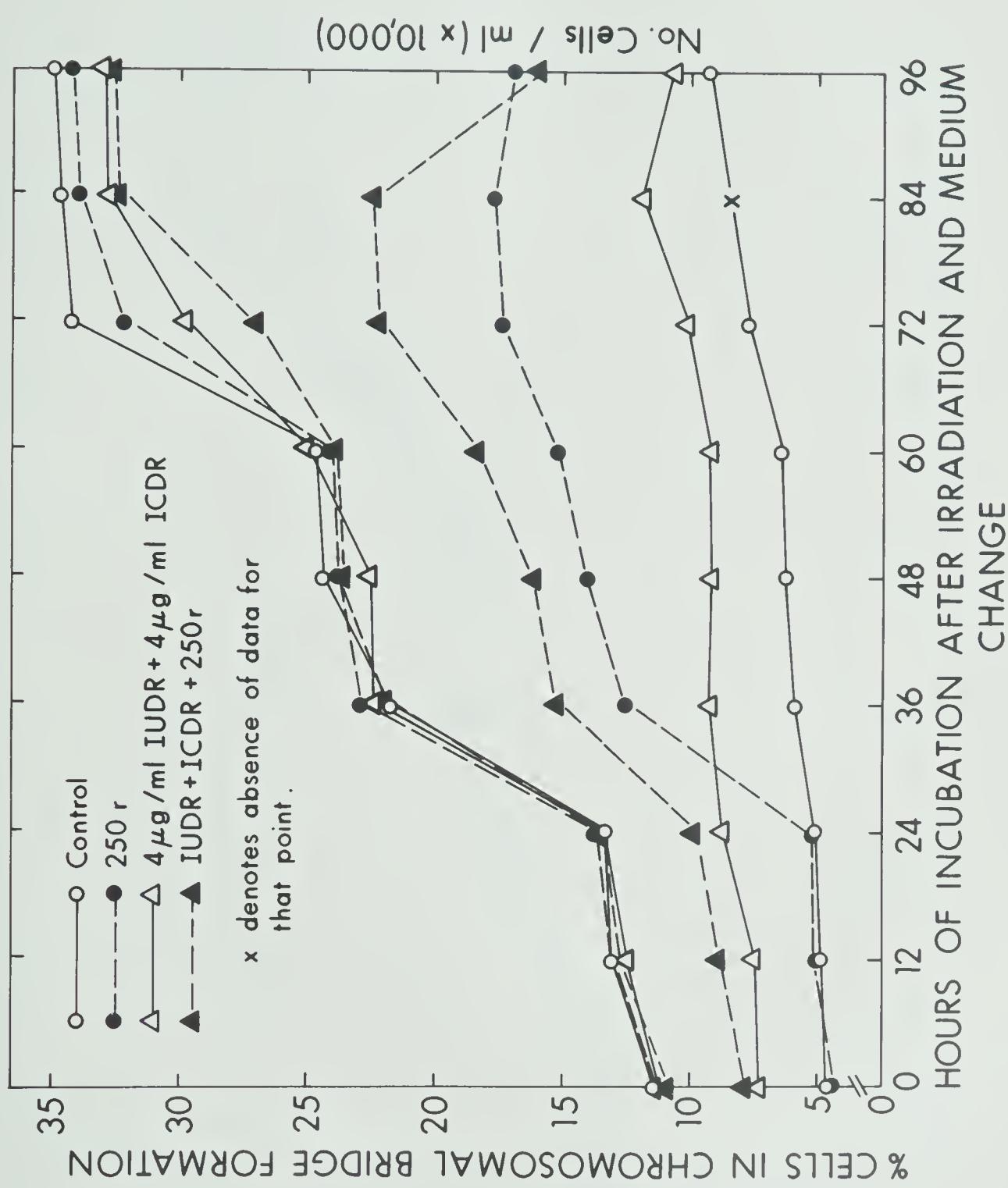
FIG. 27 A
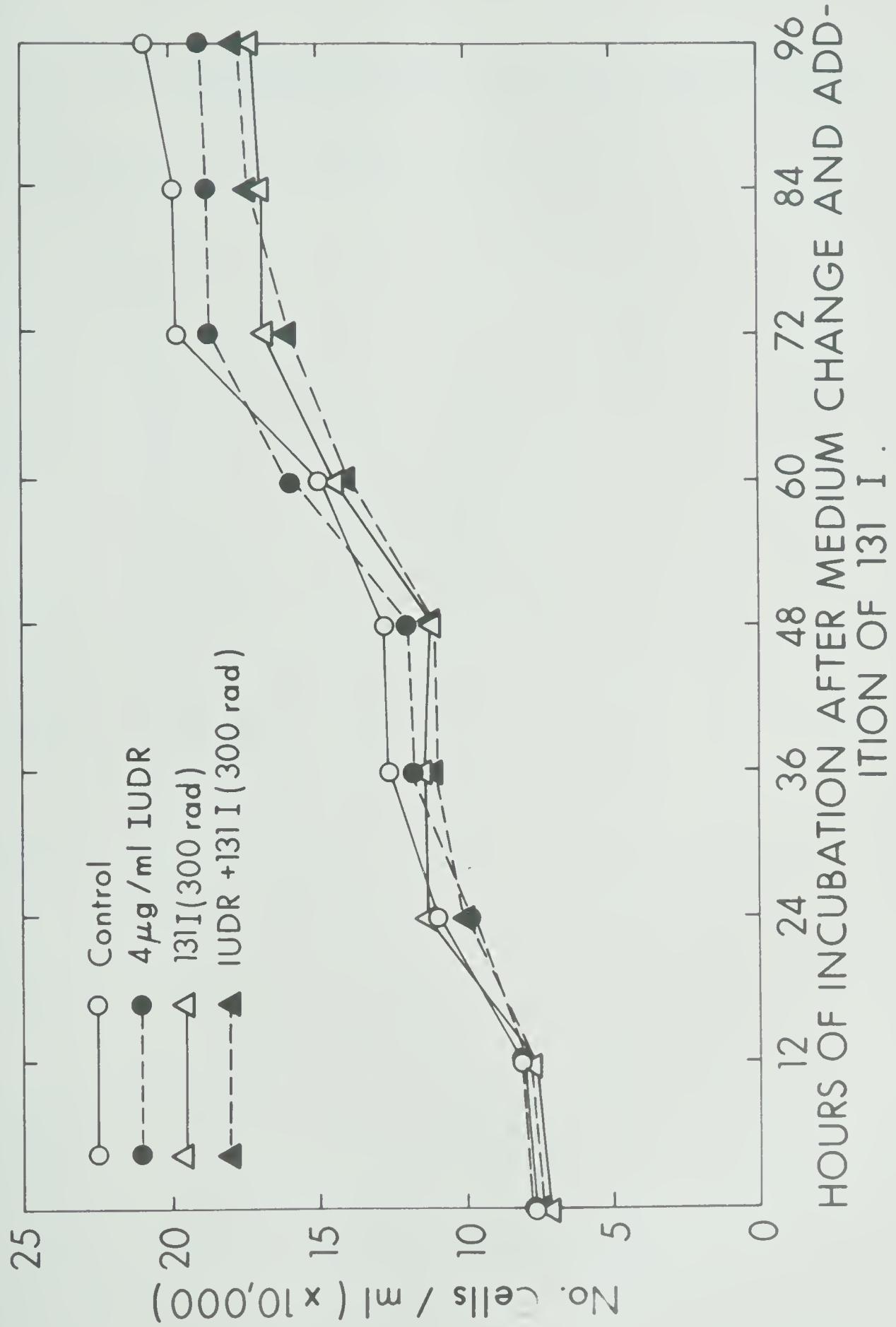
FIG. 28

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

FIG. 29

HOURS OF INCUBTION AFTER MEDIUM CHANGE AND ADD-ITION OF 131 I.

○ Control

● 4 μg/ml IUDR

△ 131 I (300 rad)

▲ IUDR + 131 I (300 rad)

x Denotes absence of data

-95-

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

FIG. 30A

HOURS OF INCUBATION AFTER MEDIUM CHANGE AND ADDITION OF 131 I.

FIG. 30 B

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

HOURS OF INCUBATION AFTER MEDIUM CHANGE AND ADD-
ITION OF 131 I.

250 r
IUDR +250 r
131I (300 rad)
IUDR +131 I (300 rad)
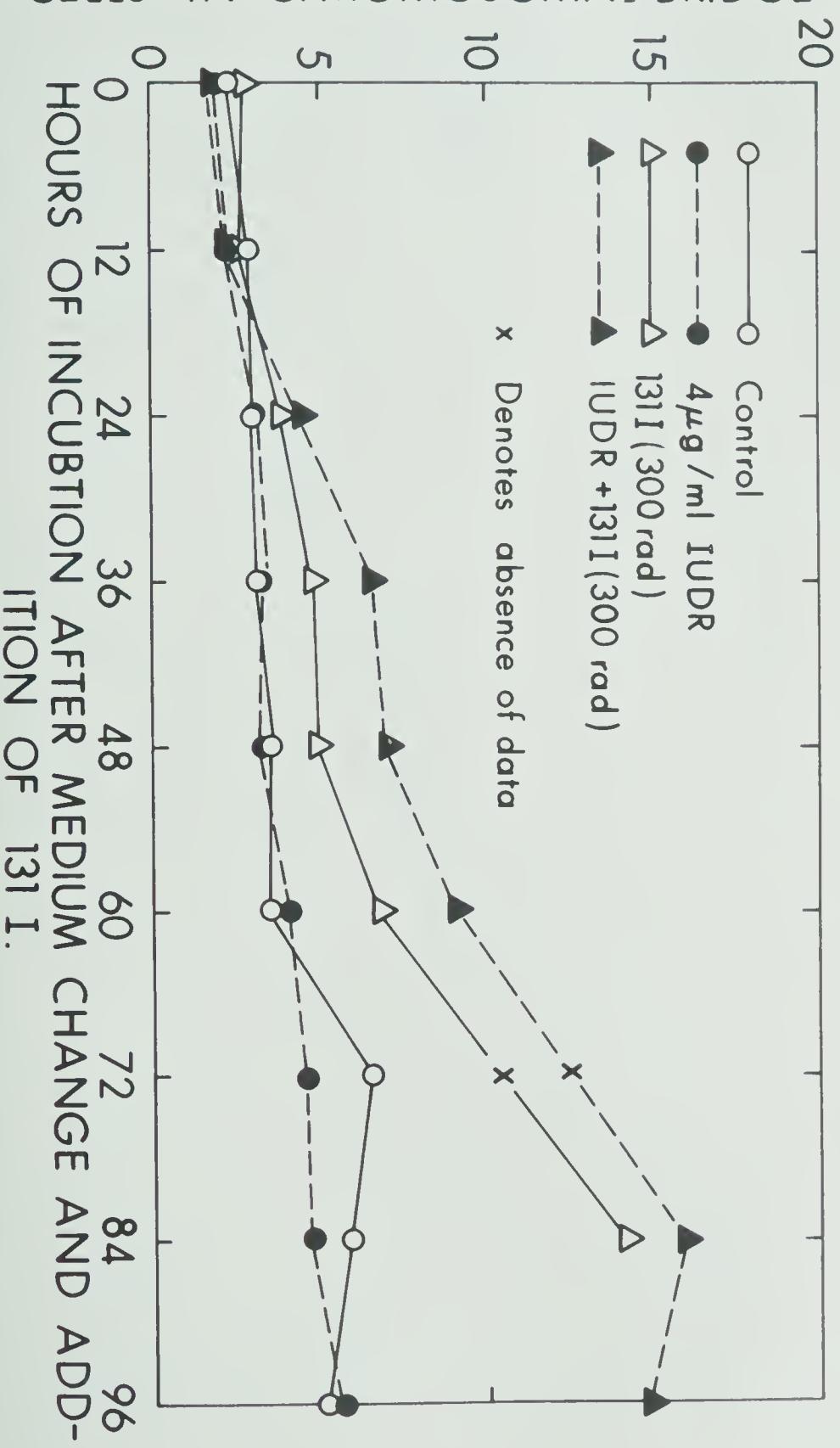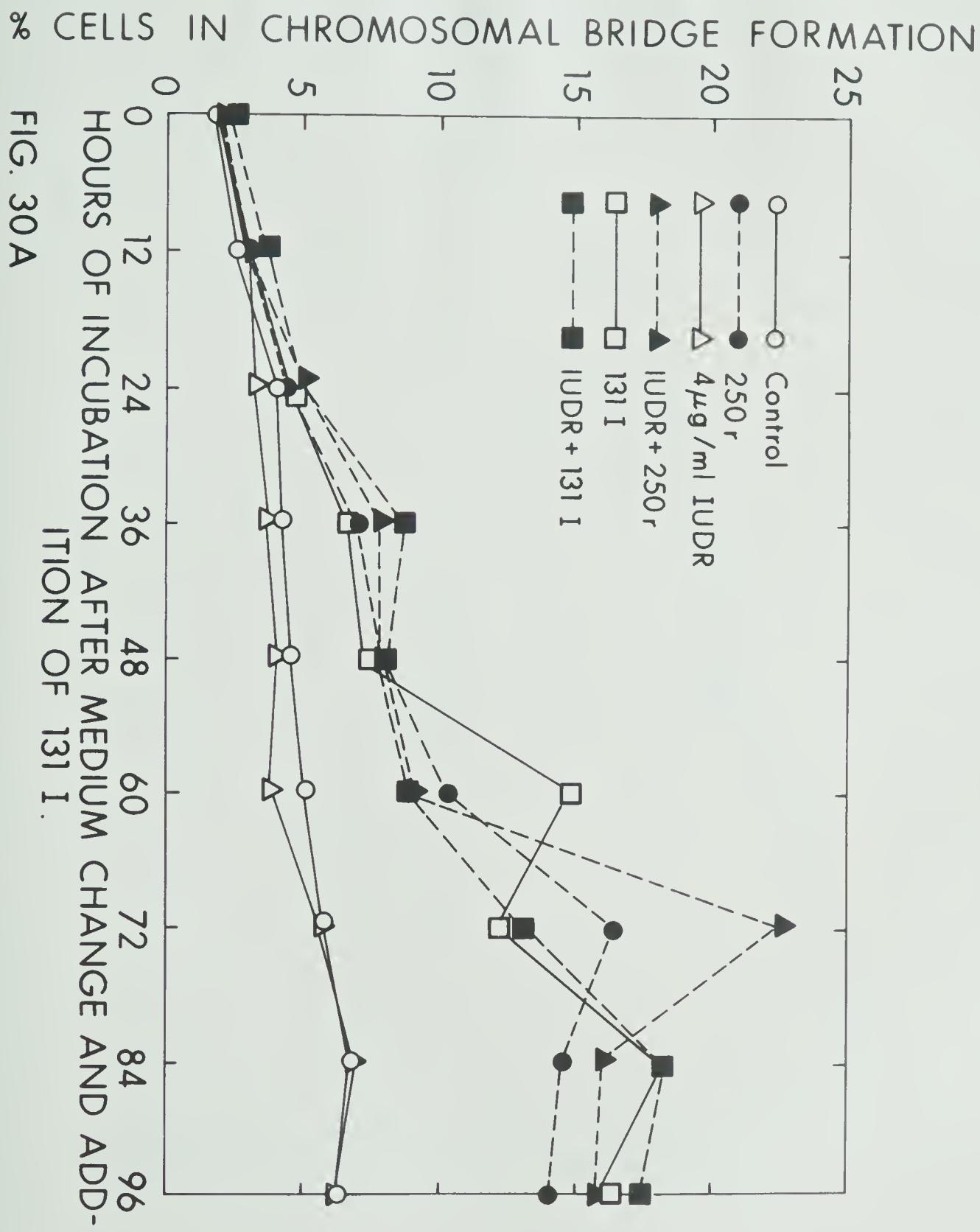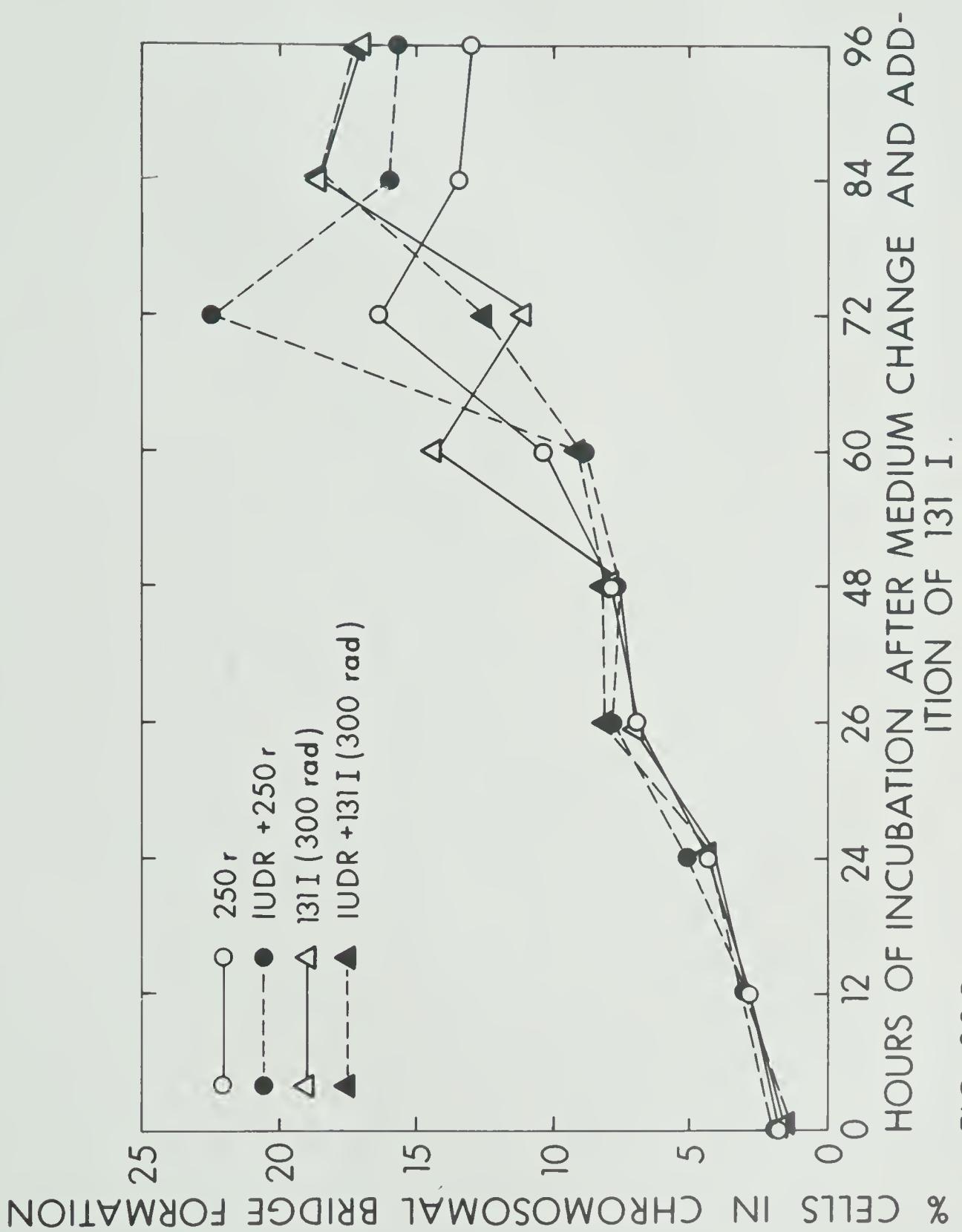
Fig. 31. A composite graph of Figs. 28 and 29 demon-
strating the dependence of chromosomal
bridge formation on mitotic activity.

FIG. 31

Legend:
- ○ Control
- ● 4 μg/ml IUDR
- △ 131 I (300 rad)
- ▲ IUDR + 131 I (300 rad)

x Denotes absence of data for that point.

% CELLS IN CHROMOSOMAL BRIDGE FORMATION

HOURS OF INCUBATION AFTER MEDIUM CHANGE AND ADD-ITION OF 131 I.
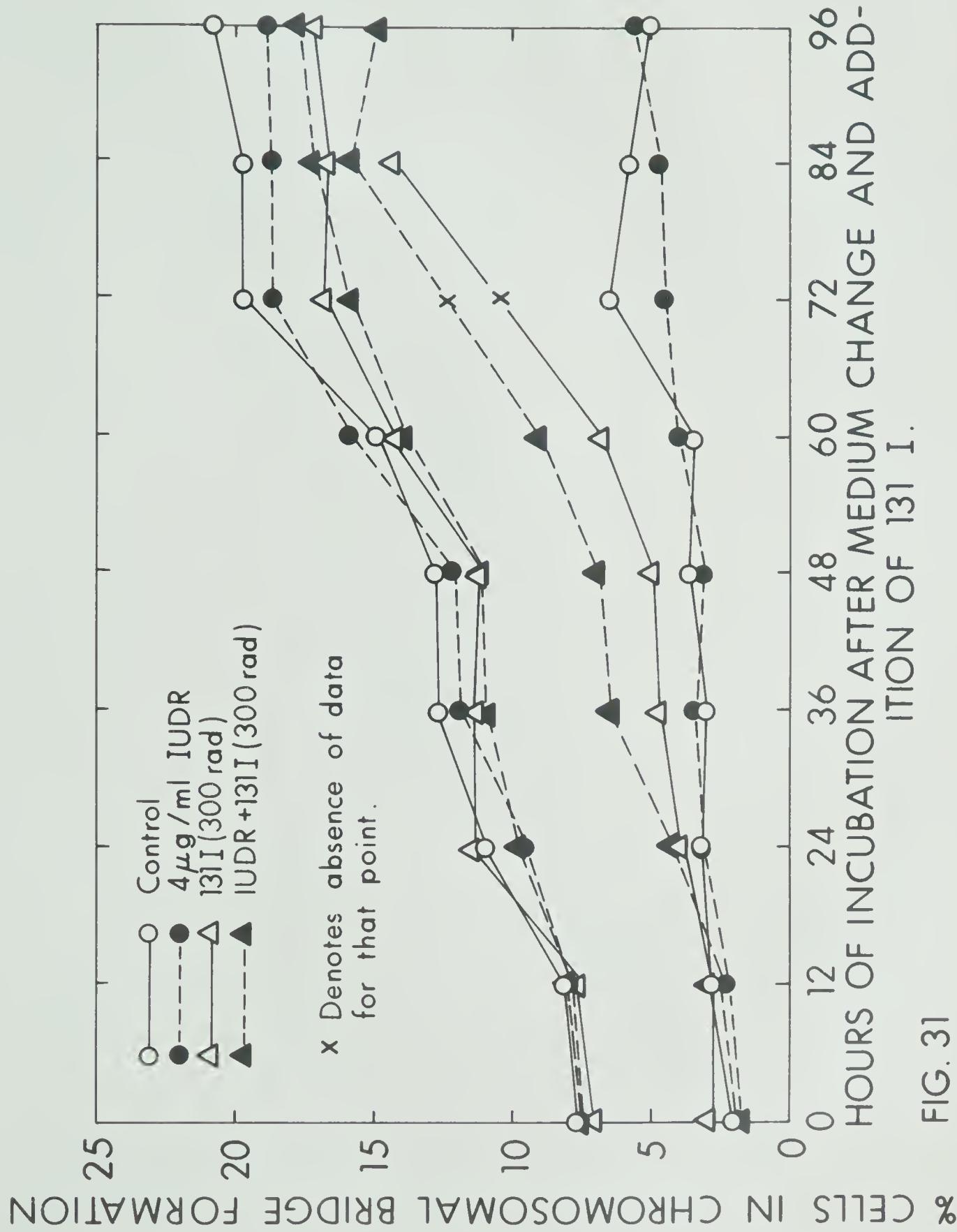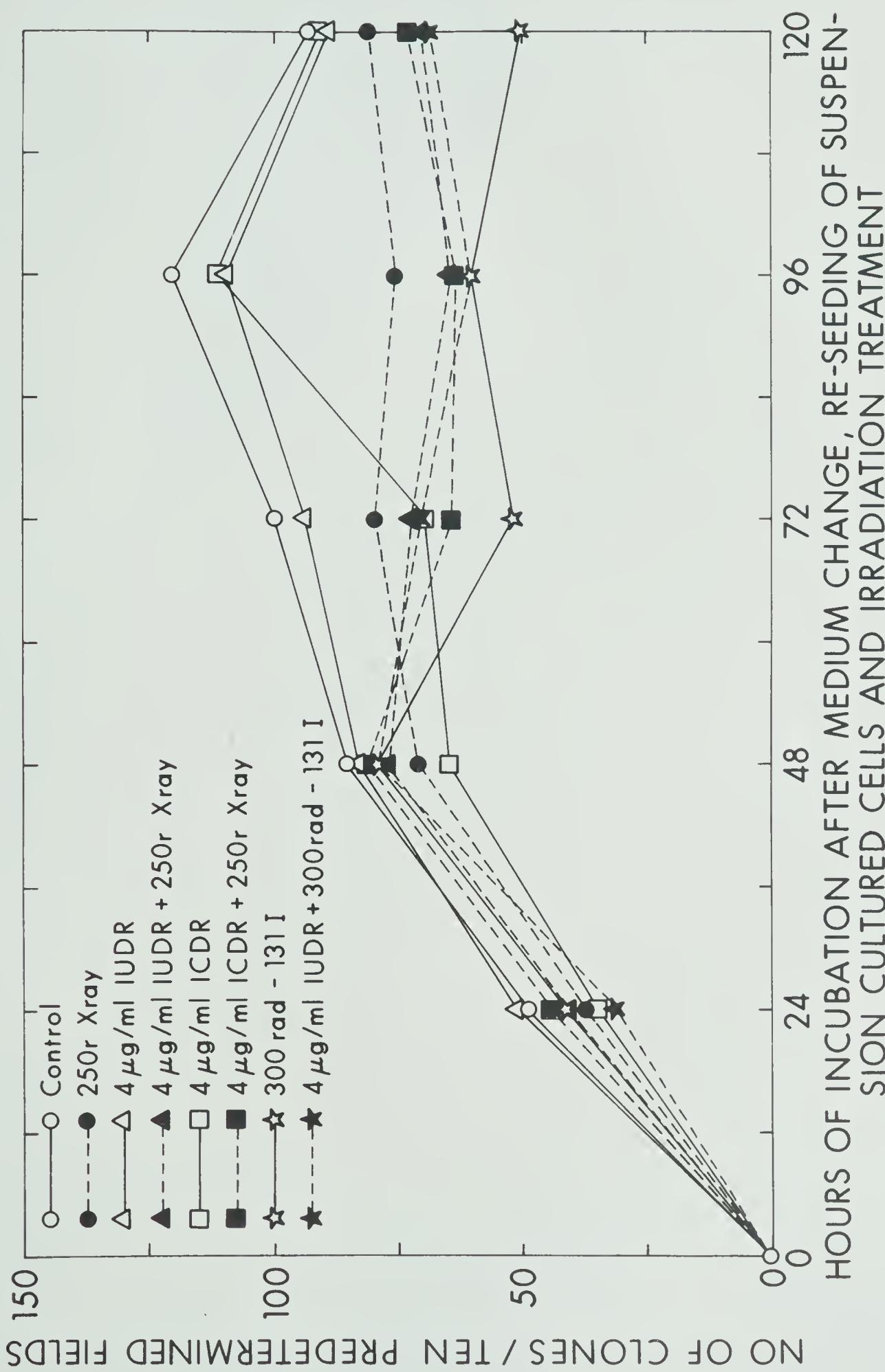
FIG. 32

PLATE: I

Normal epithelial calf thyroid cells in interphase.

Mag.   X2800.

      nl   -   nucleolus

       n   -   nucleus
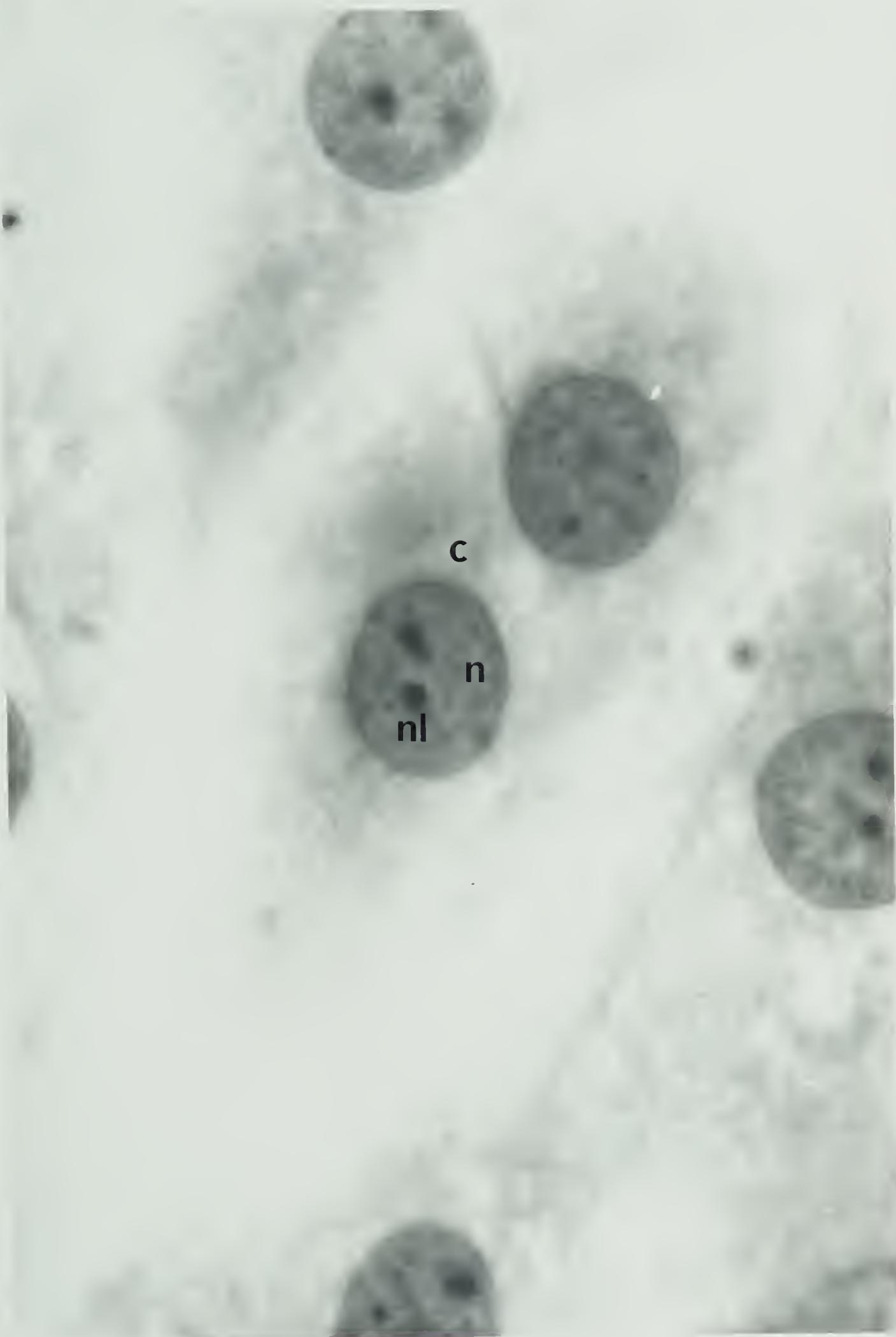
       c   -   cytoplasm

PLATE: II

Normal epithelial calf thyroid cell in prophase.

Mag.   X2800.

    n - nucleus
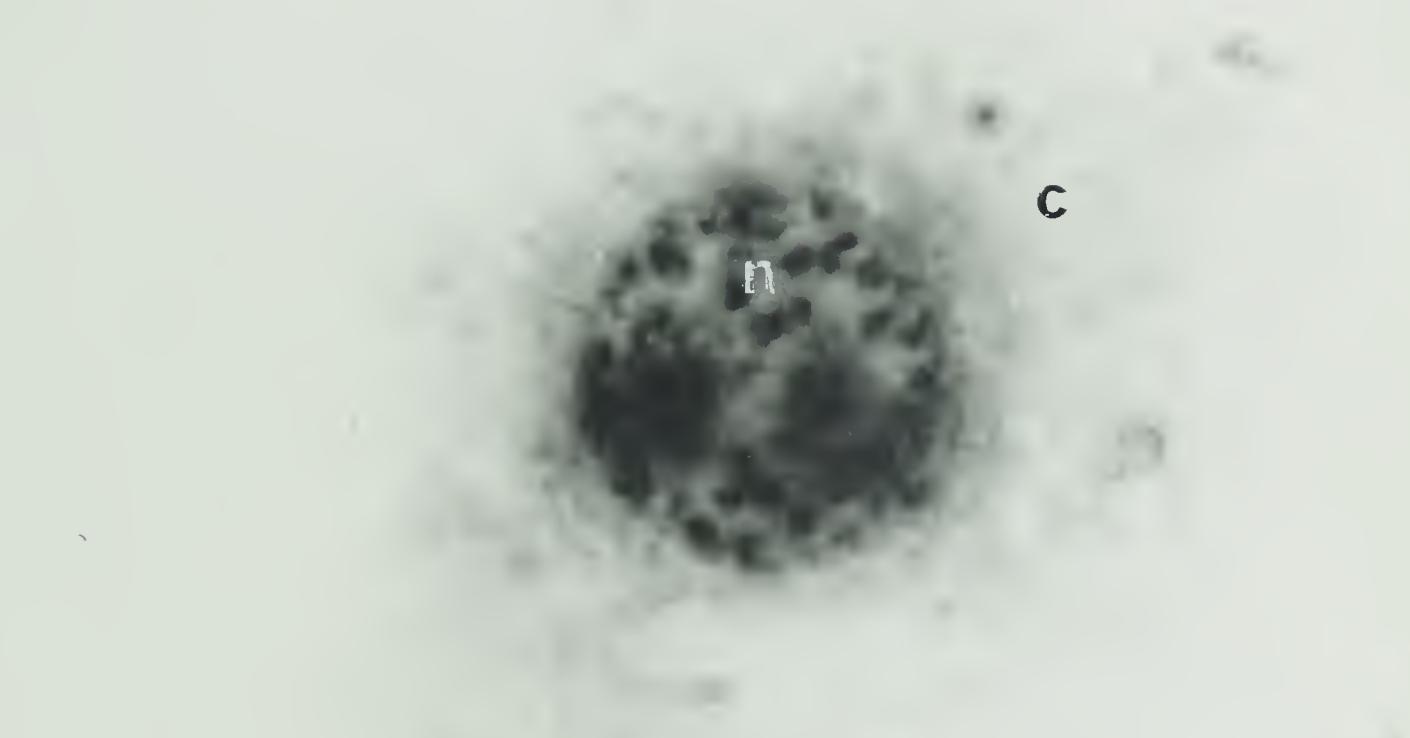
    c - cytoplasm

PLATE: III

Epithelial calf thyroid cell in metaphase.

Mag.   X2800.

c   -   cytoplasm

PLATE: IV

Epithelial calf thyroid cell in metaphase. Note
what appears to be a di- or poly-centric chromosome.

Mag. X2800.

   c - cytoplasm

.
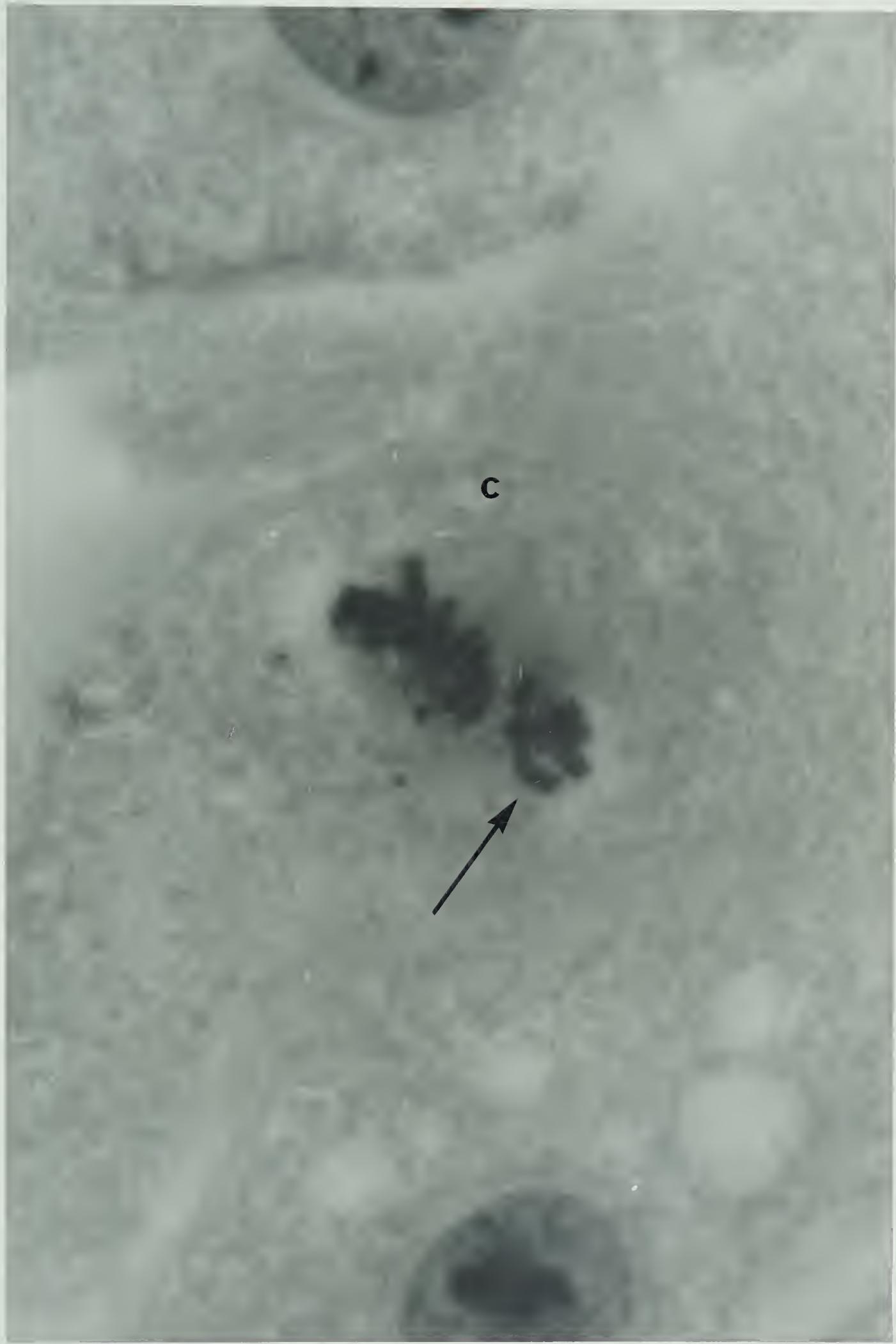
PLATE: V

Normal epithelial calf thyroid cell in late anaphase.

Mag. X2800.

nl -   nucleolus
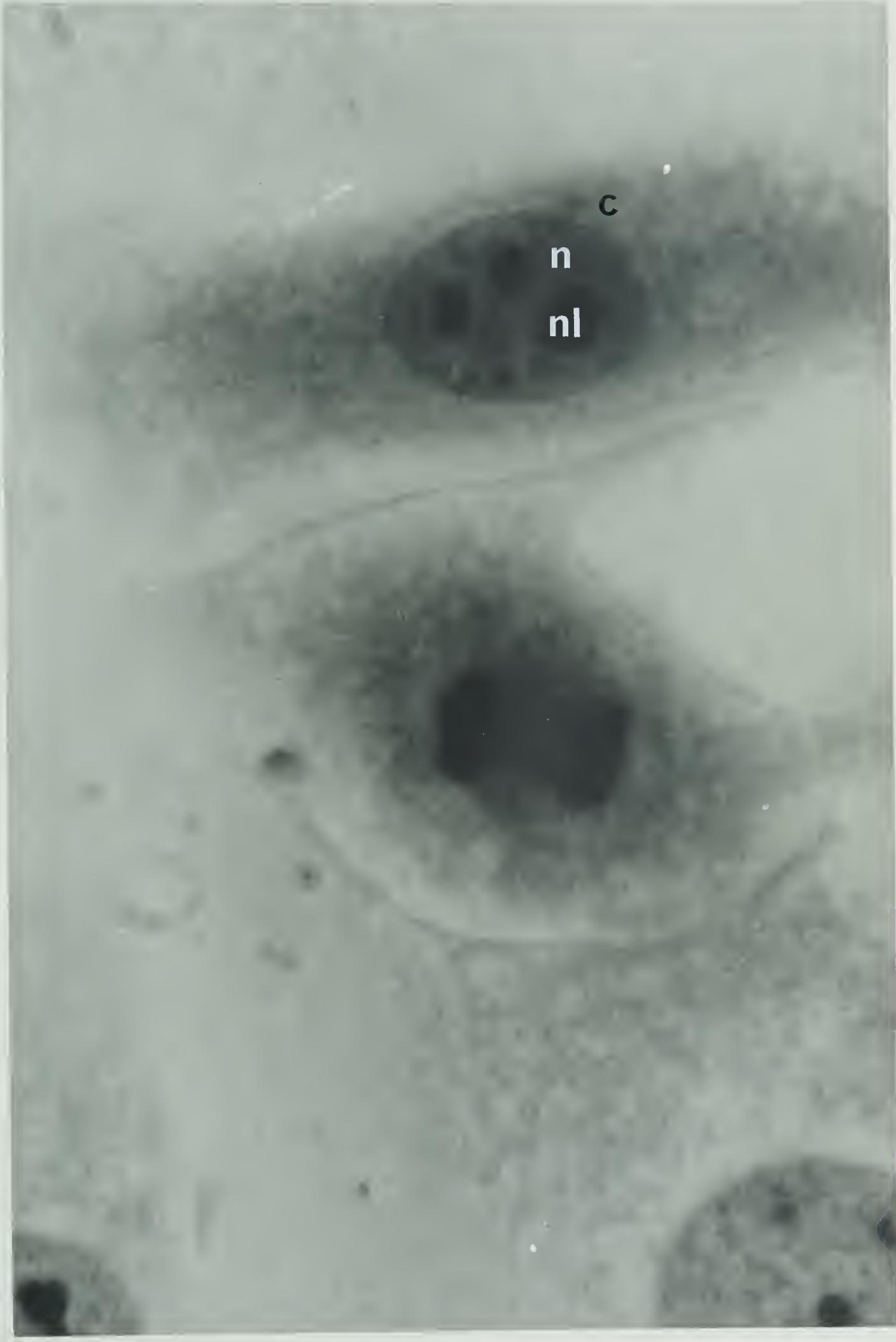
n -   nucleus

c -   cytoplasm

PLATE: VI

Epithelial calf thyroid cell. Note the
invaginated nucleus and chromatin bridge.

Mag. X2800.

  nl - nucleolus

   n - nucleus

   c - cytoplasm

.

.
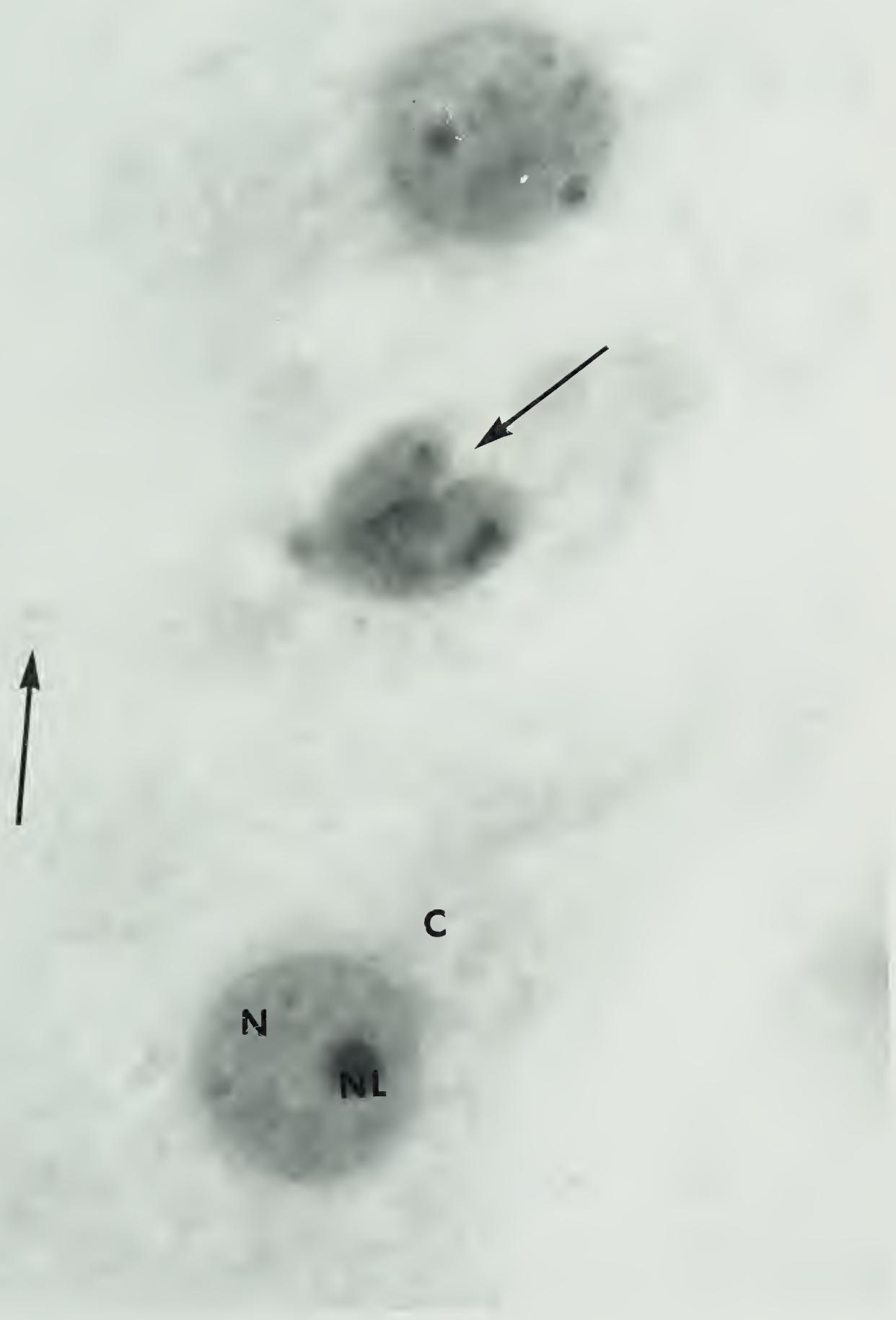
PLATE: VII

Epithelial calf thyroid cells. The upper cell
is in prophase. Note the incomplete and unequal
division of the lower cell.

Mag. X2800.

n - nucleus

c - cytoplasm

.
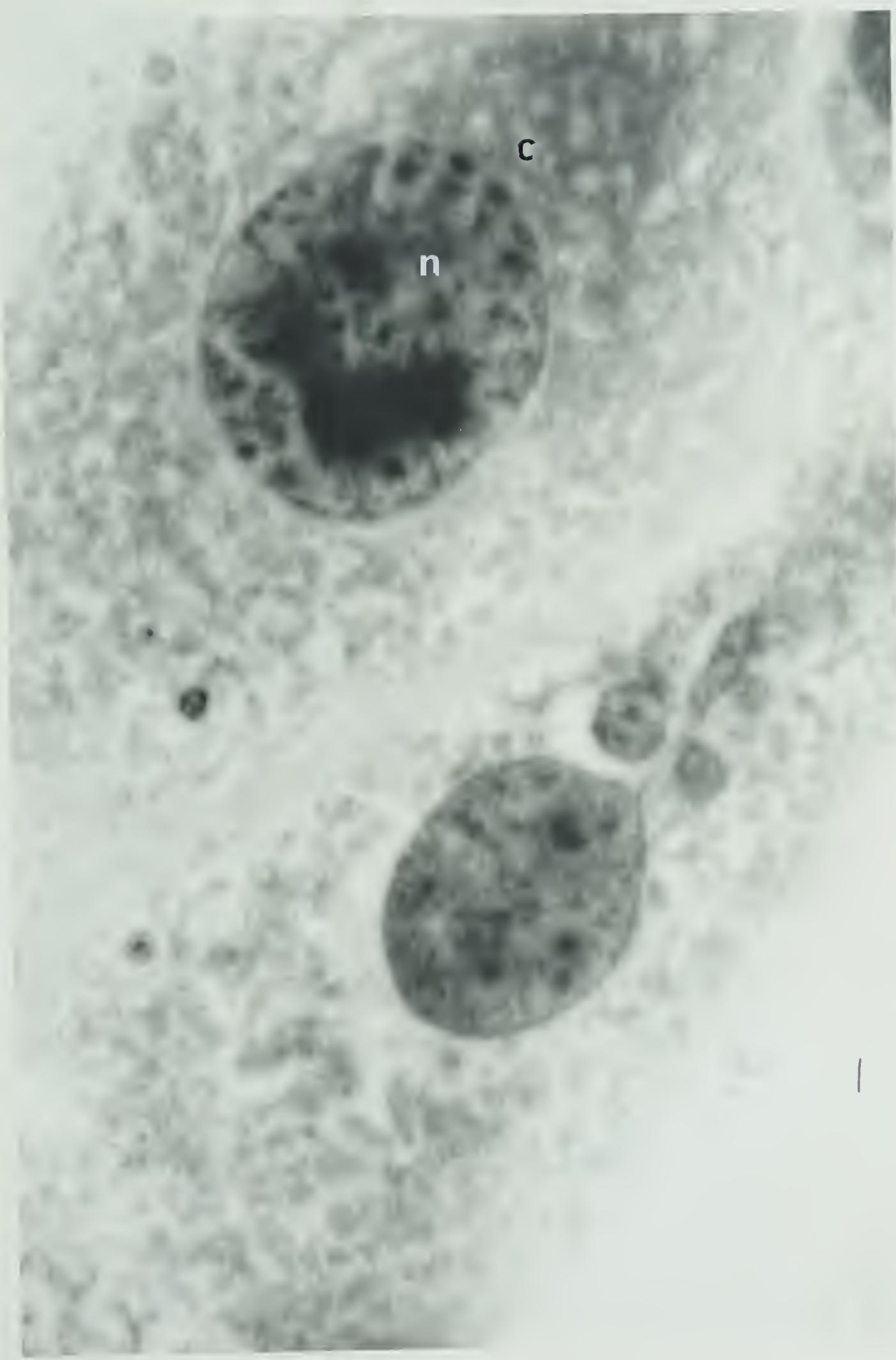
PLATE: VIII

Epithelial calf thyroid cells. Note the
bridge between two daughter cells.

Mag. X2800.

    nl - nucleolus
     n - nucleus
     c - Cytoplasm
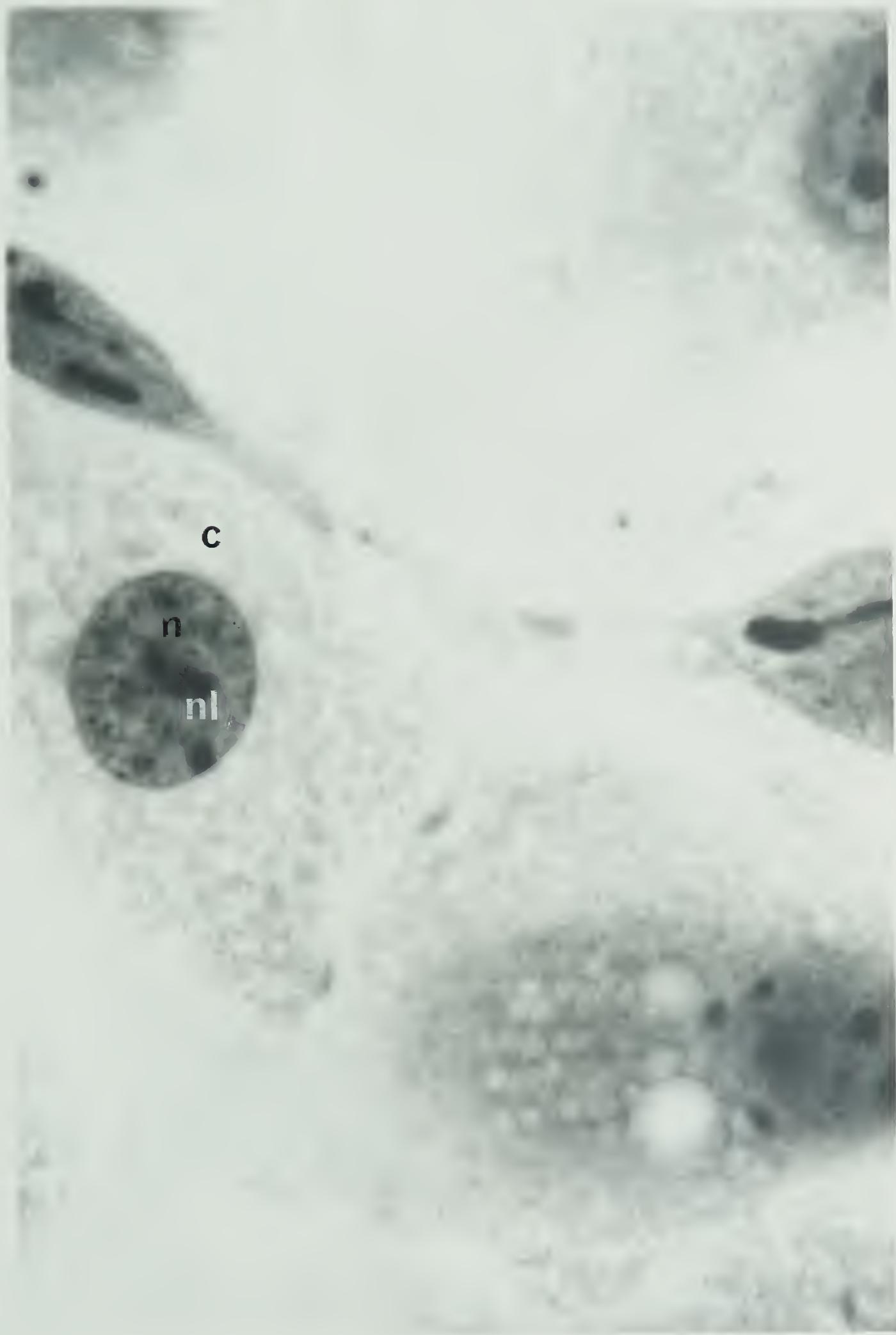
PLATE: IX

Epithelial calf thyroid cells. Note the
classical bridge between the two daughter
cells.

Mag. X2800.

   nl -   nucleolus

    n -   nucleus
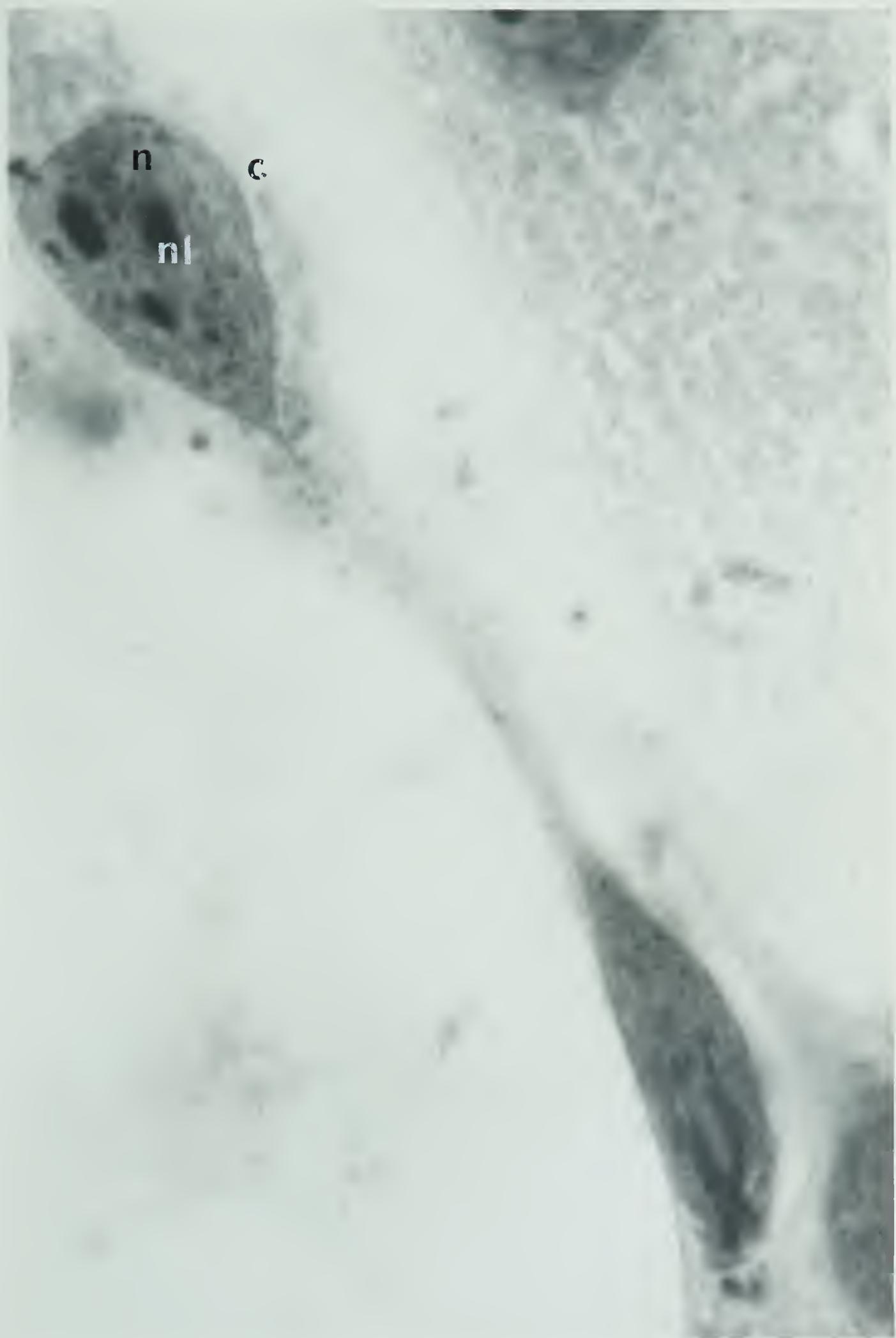
    c -   cytoplasm

PLATE: X

Epithelial calf thyroid cells. Note the chromatin
bridge between the two daughter cells.

Mag. X2800.

    nl - nucleolus

     n - nucleus

     c - cytoplasm

PLATE: XI

Epithelial calf thyroid cells. Note the chromatin
bridges between the two daughter cells.

Mag. X2800.

    n - nucleus
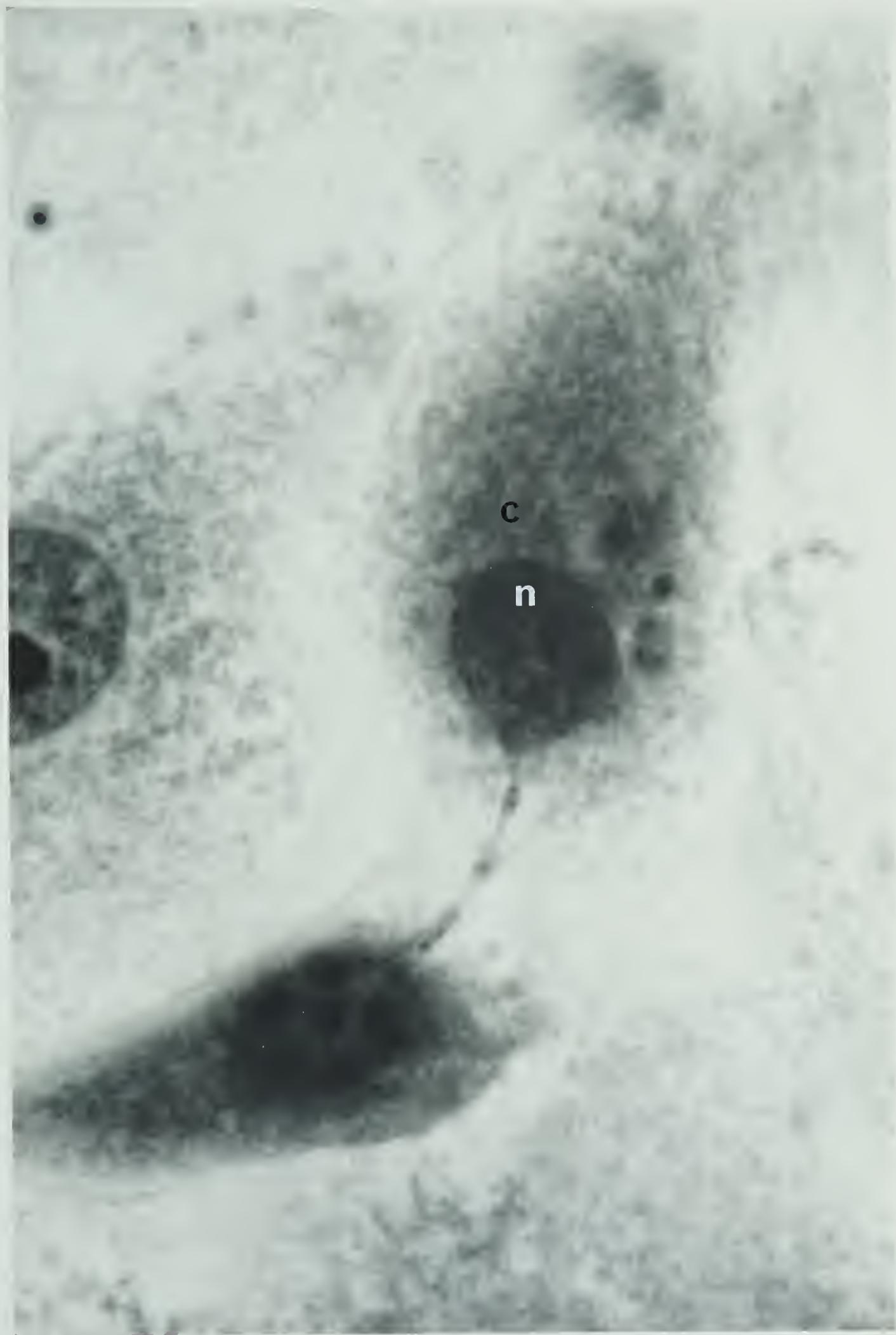
    c - cytoplasm

PLATE: XII

Normal epithelial calf kidney cell in prophase.

Mag. X2800.

    n -   nucleus
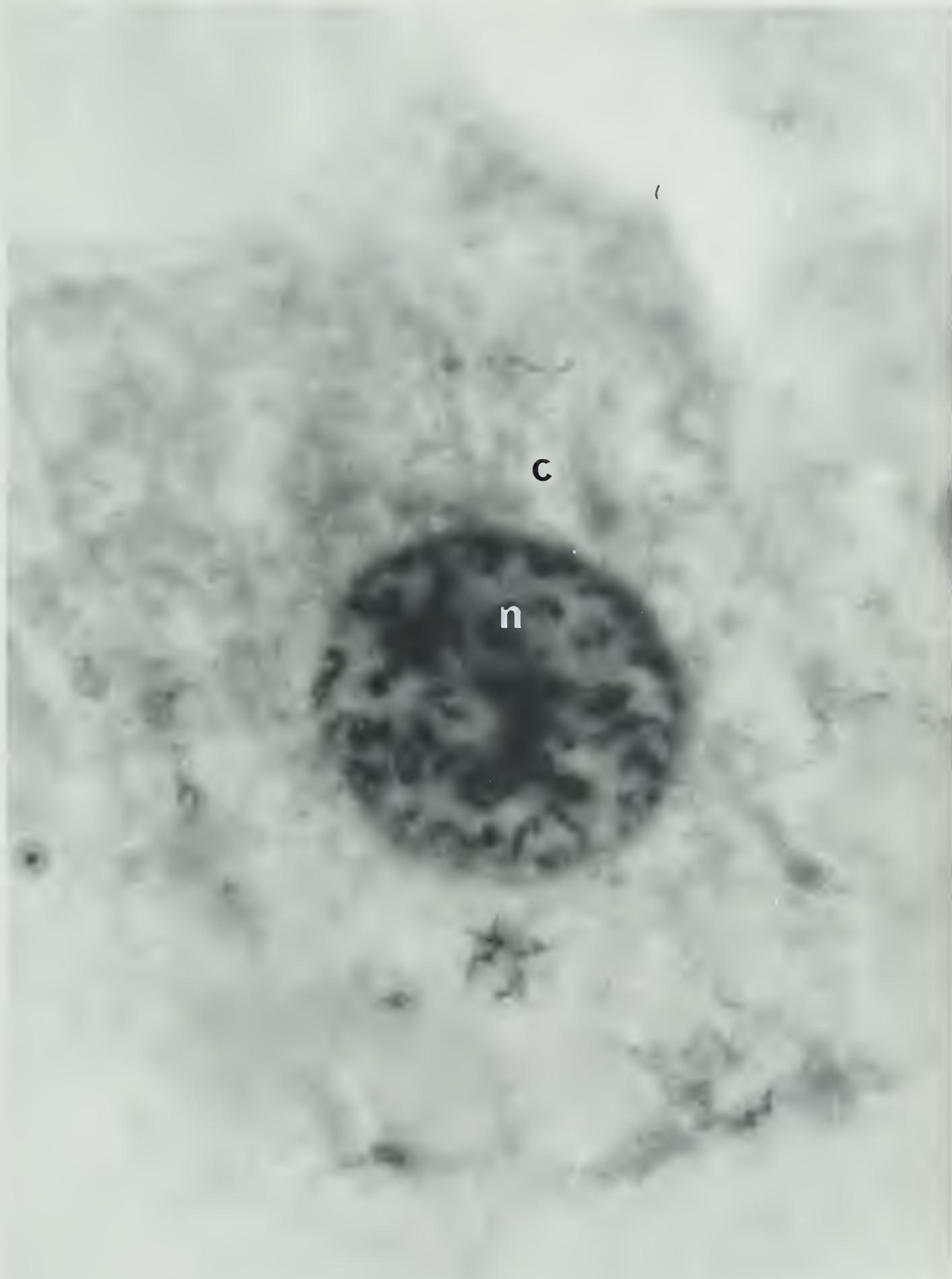
    c -   cytoplasm

PLATE: XIII

Epithelial calf kidney cell in metaphase.
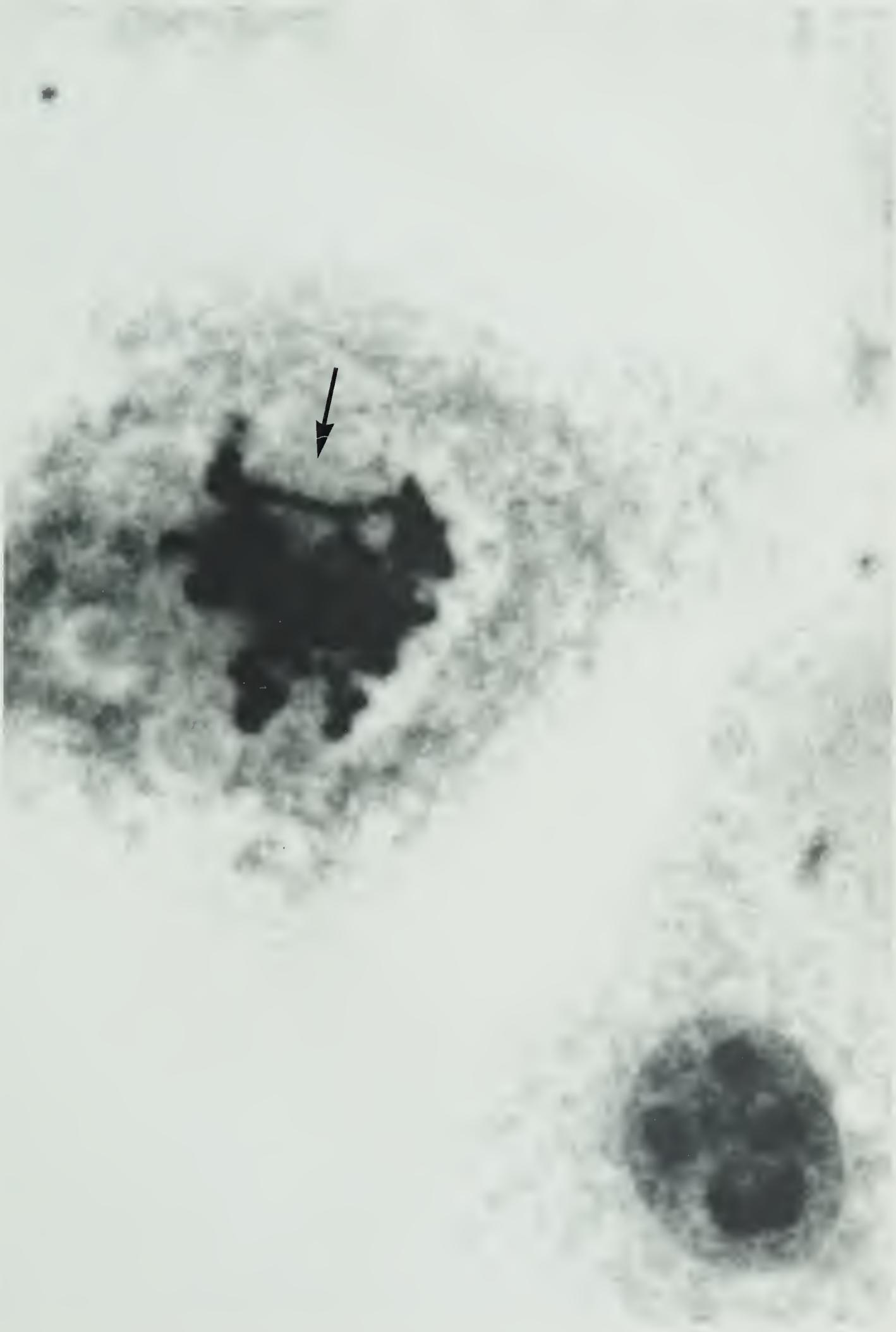
Mag. X2800.

PLATE: · XIV

Normal epithelial calf kidney cell in late anaphase.

Mag. X2800.

c - cytoplasm

c
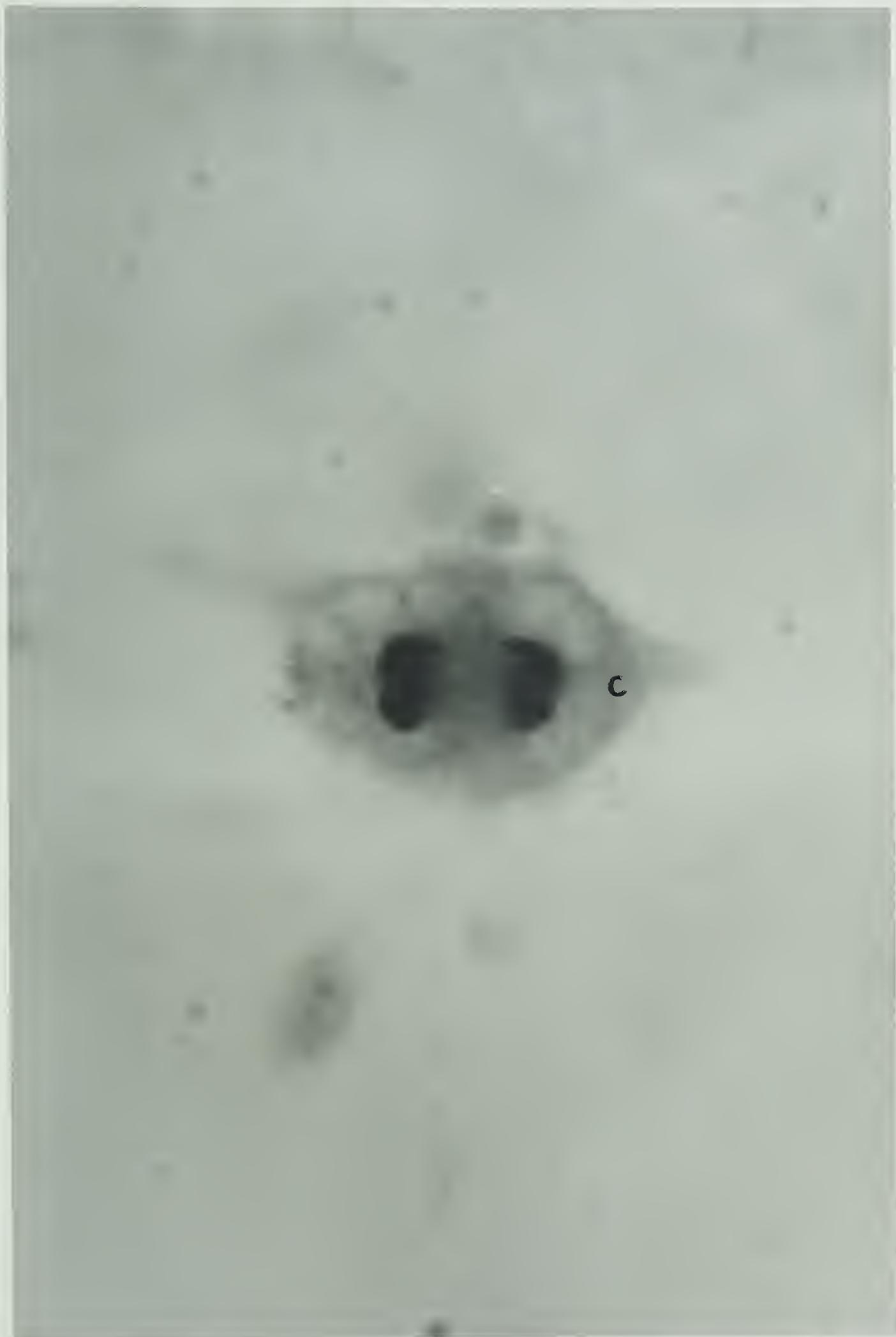
PLATE: XV

Normal epithelial calf kidney cell in late anaphase.

Mag. X2800.
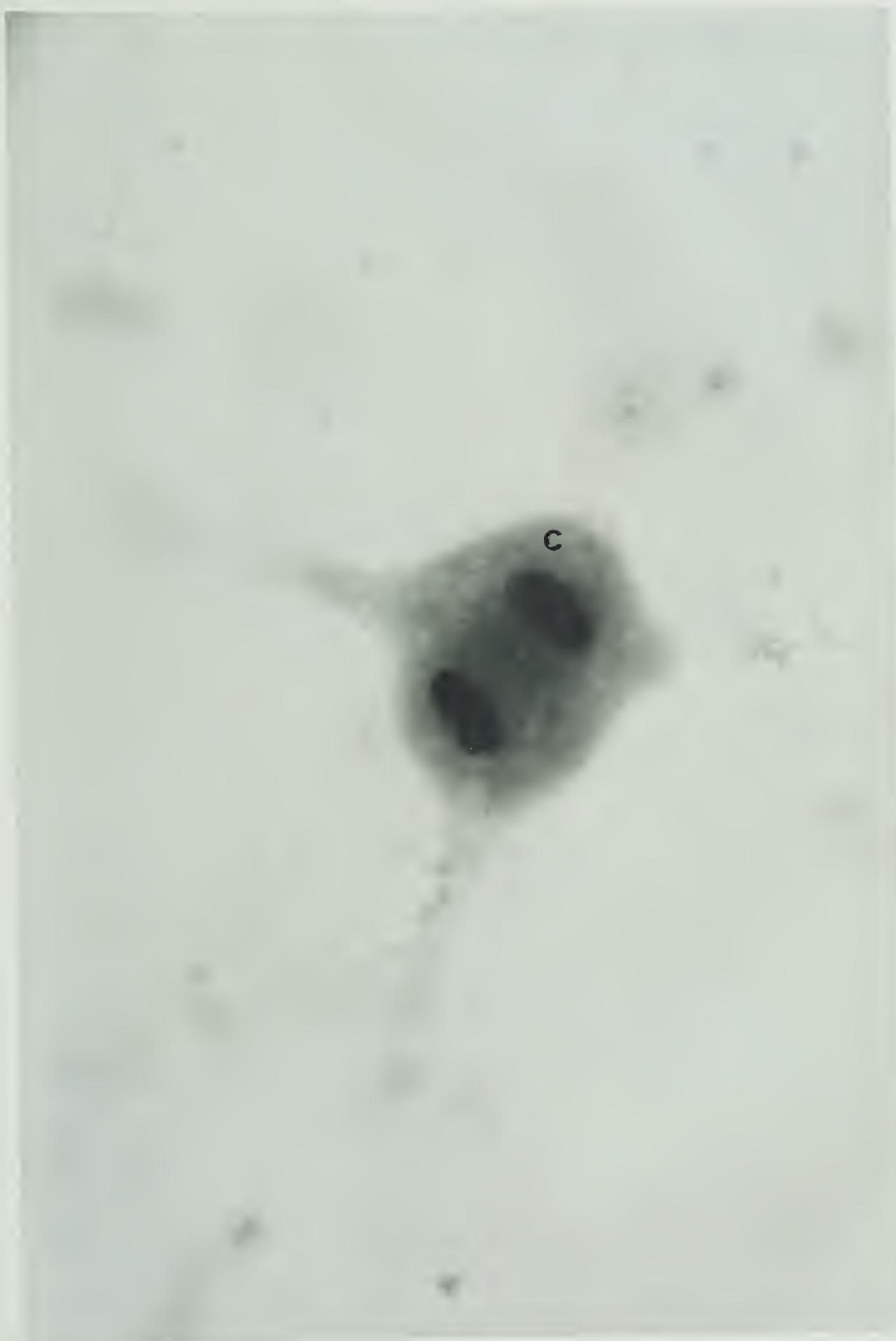
c - cytoplasm

PLATE: XVI

Epithelial calf kidney cell in telophase.
Note the chromatin bridge.

Mag. X2800.

c   -   cytoplasm
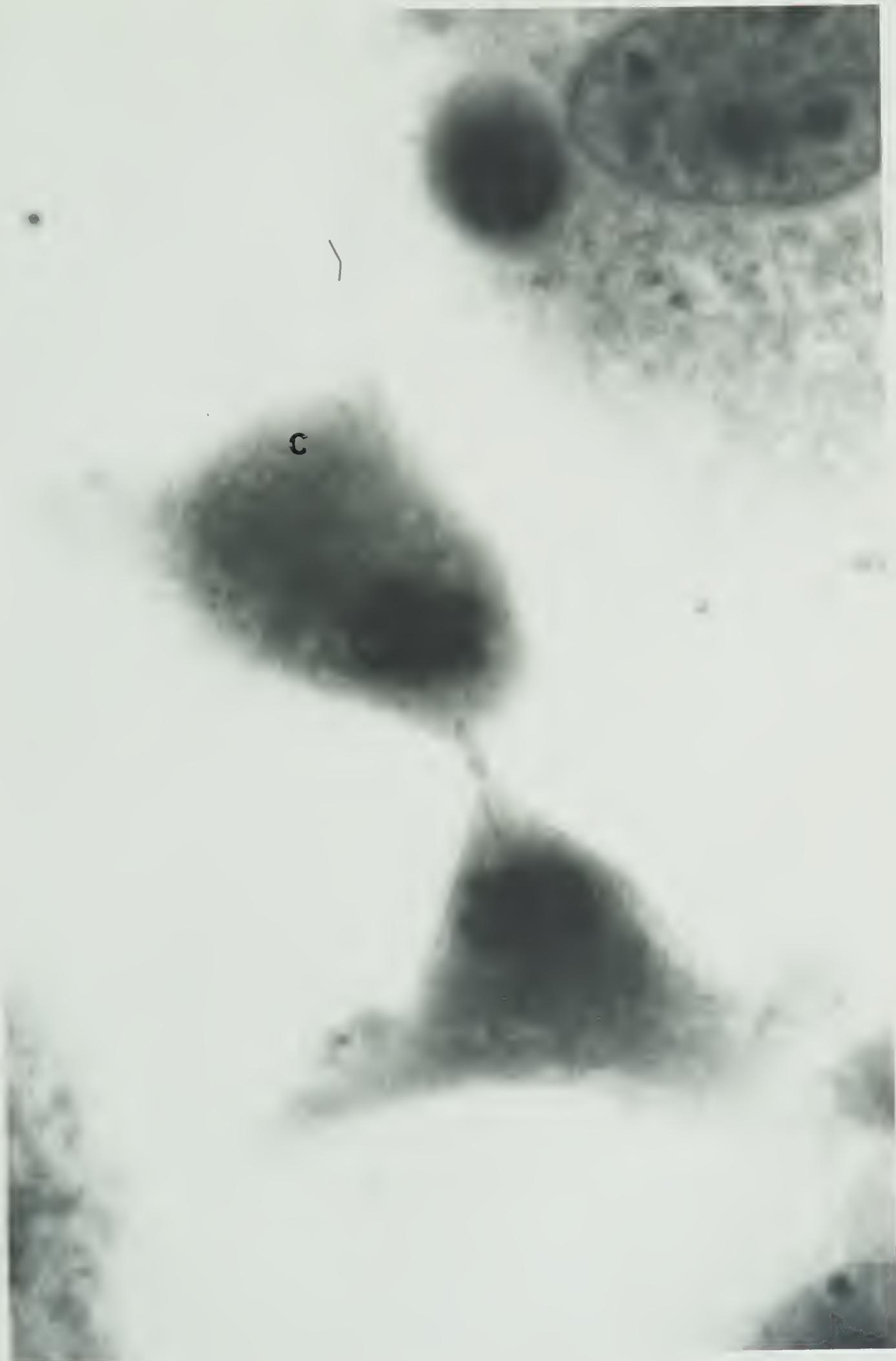
PLATE: XVII

Normal epithelial calf kidney cell in telophase.

Mag. X2800.

     n - nucleus
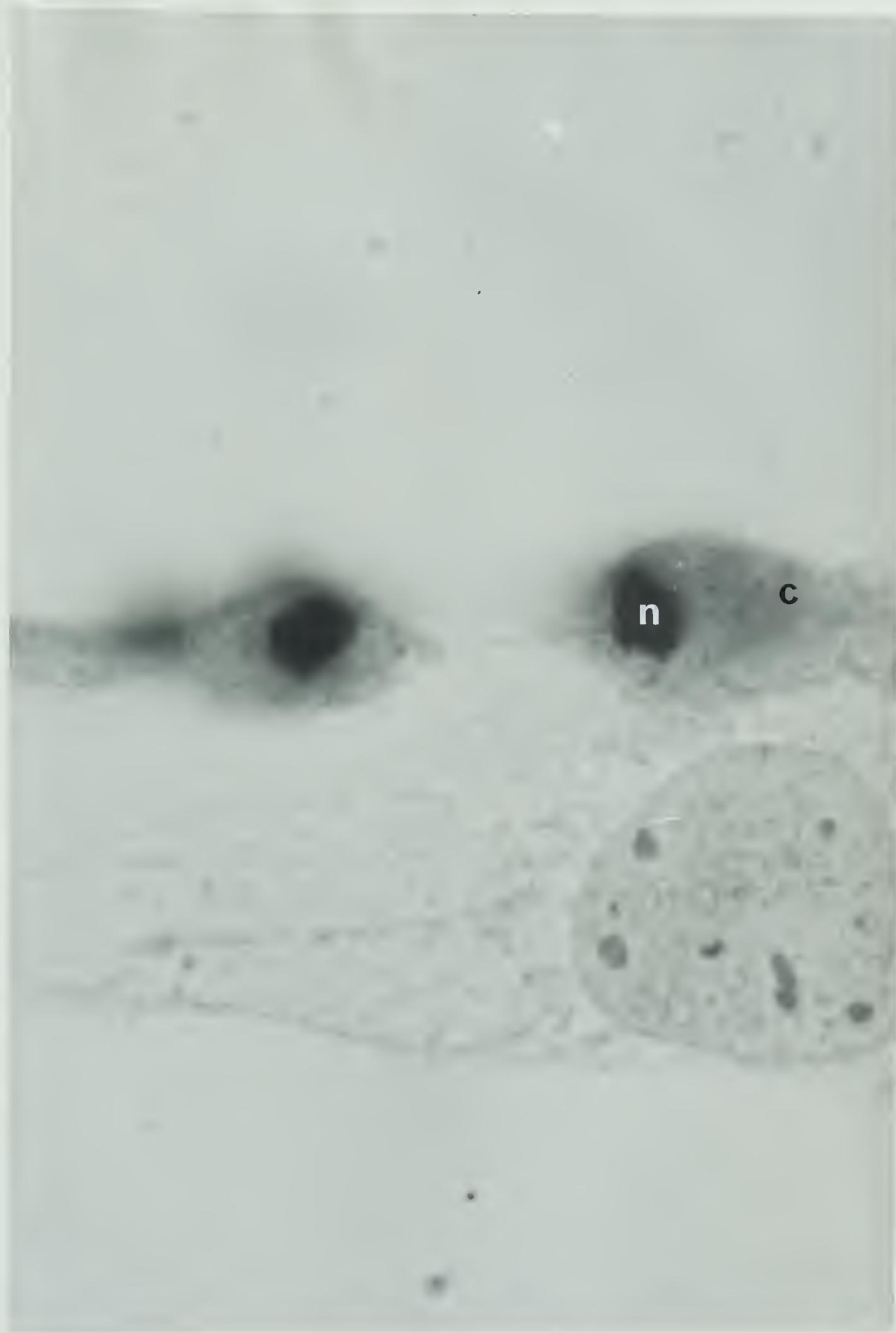
     c - cytoplasm

PLATE: XVIII

Normal epithelial calf thyroid cells exposed to 125I-UDR. Note the dense 125I-labelling above the nuclei.

Mag. X2800.

      n -   nucleus
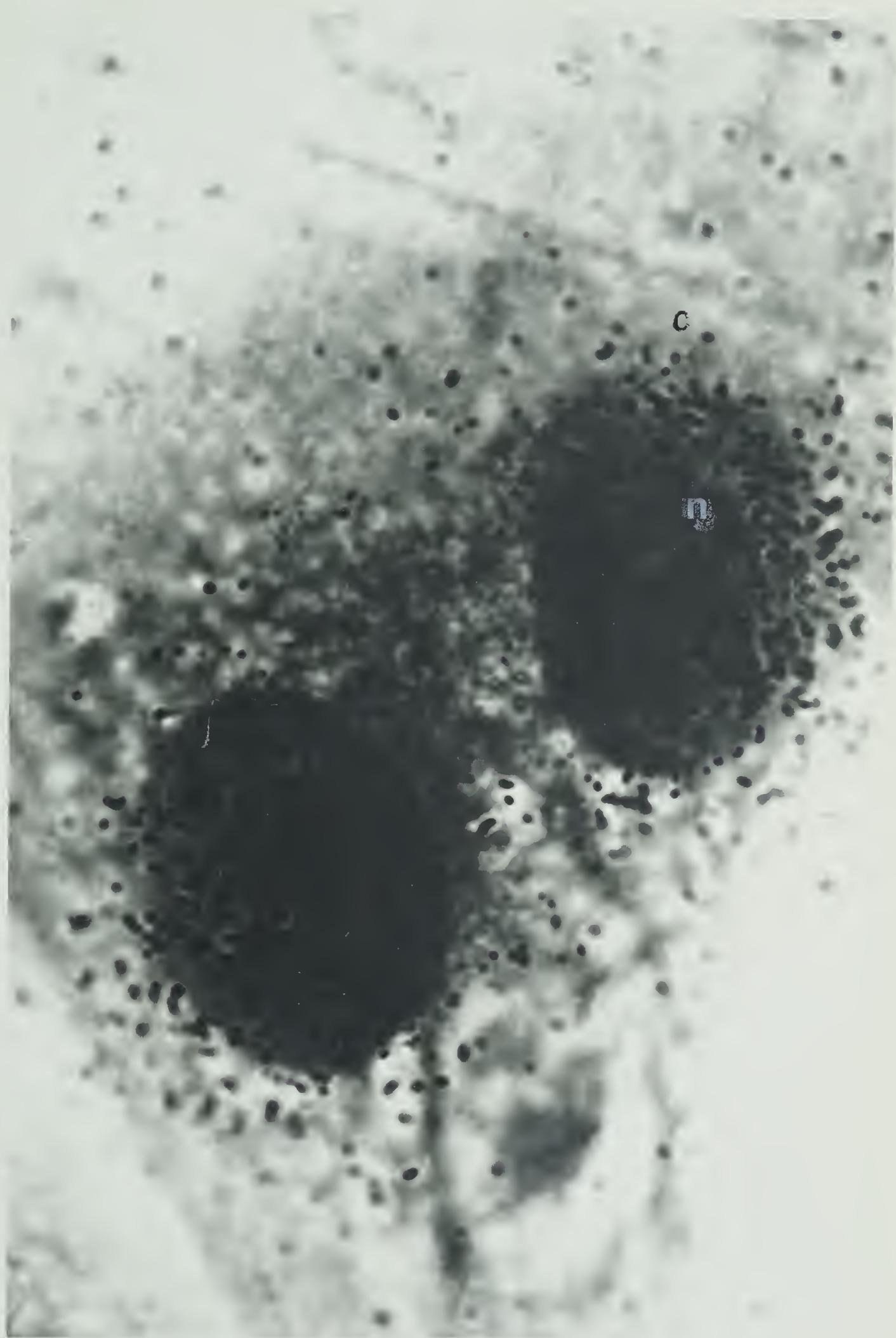
      c -   cytoplasm

PLATE: XIX

Normal epithelial calf thyroid cell exposed to 125I-
UDR. Note the 125I-labelling above the nucleus.

Mag. X2800.

       n - nucleus
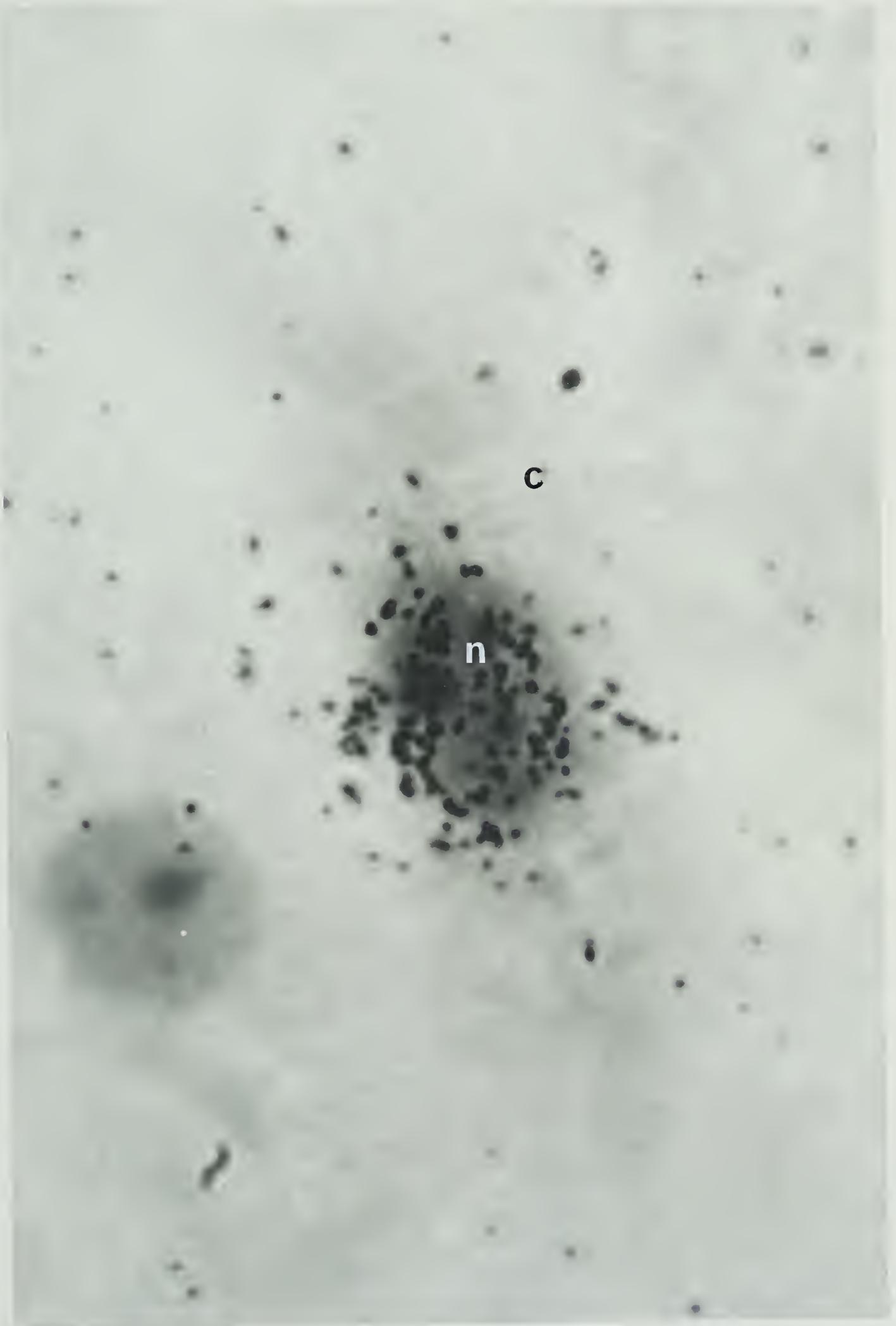
       c - cytoplasm

PLATE: XX

Normal epithelial calf thyroid cells exposed to
125I-UDR. Note the 125I-labelling above the two
telophase nuclei. (not in focus)

Mag. X2800.

    n -   nucleus

    c -   cytoplasm
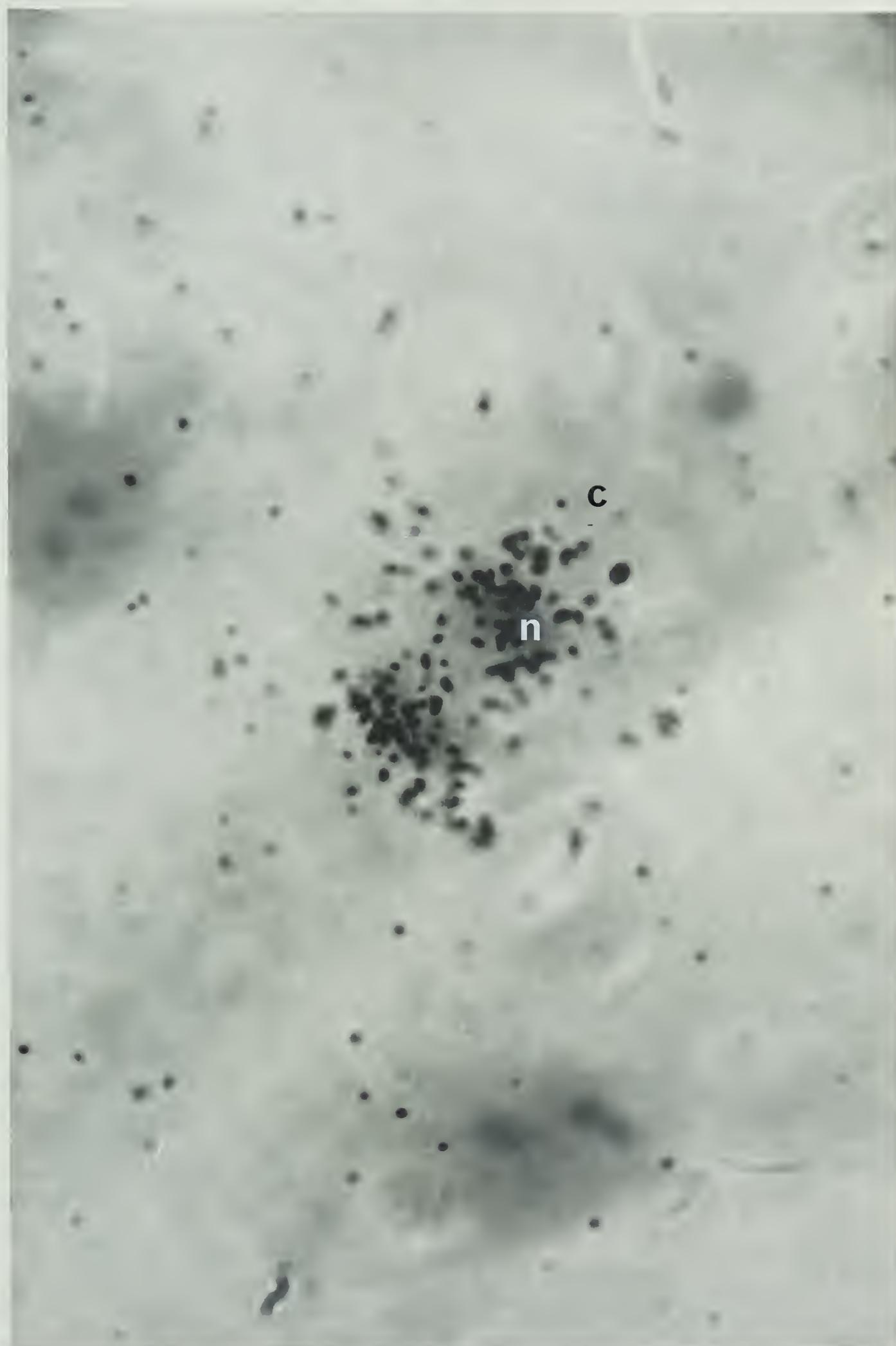
DISCUSSION

1. General

The development of more efficient means by which
malignant tumors may be arrested or eradicated has generated
considerable interest. With this in mind, two lines of
development, (a) chemotherapy, and (b) radiotherapy, have
been under active investigation, the latter dating back
to the findings of Bergonie and Tribondeau (1906). Inves-
tigations have in fact, led to studies of the combined use
of radio and chemotherapy and their possible synergistic
consequences. With the recent introduction of a family of
compounds known as the Halogen Analogues (of the DNA bases
of Thymine and Cytosine) there has been an avalanche of
scientific literature on:

    (a)   the use of these analogues as
         a basic scientific tool for
         genetical and biochemical study.

    (b)   the exploration of the combined
         effects of the analogues and
         radiation damage in cell systems.

It is the latter avenue which has been investigated
in this thesis with the view to some possible future appli-
cation.

The premise on which this thesis is based, is
taken from the pioneer work of Greer and Zamenof (1957) which
showed that some of these analogues were incorporated into
newly replicated DNA, and that their incorporation resulted
in radio potentiation. The studies of Puck and Marcus (1956)
with mammalian cells, indicated that the genetic apparatus
was damaged by the action of x-irradiation. A consequence
of this x-irradiation was chromosomal breakage with subsequent
normal and abnormal restitution. A manifestation of the

abnormal restitution of chromosomes has been shown by
Whitfield, Rixon and Rhynas (1959), in their work with
L strain mouse cells and in the present study with thy-
roid tissue. Kihlman's (1963) finding, that in Vicia
faba, chromatid breakage due to x-rays was enhanced by
prior exposure of the plant to IUDR, should be noted.
Ragni and Szybalski (1962) whilst studying human cells,
implied that halo-analogues enhanced scission of one of
the DNA strands, and this could lead to eventual cell
lethality. Dewey and Humphrey (1965) have also shown that
BUDR enhanced x-ray breakage of chromosomes in Chinese
hamster cells. It should be added that Chu (1965a) noted
that UV-induced chromosomal breakage is enhanced by IUDR
in Chinese hamster cells.

X-irradiation induced chromosomal breakage,
the enhancement of chromosomal breakage by prior exposure
of the cells to IUDR, and a cytological means by which
this x-irradiation damage could be detected, made investi-
gation of the thyroid cell system feasible.

2. Cytological Evaluation of X-irradiation Damage

The present study shows that CB formation in
thyroid cells is increased by x-ray exposure, the greatest
effect being observed following 250R. Higher doses (500
and 1000R) were less effective. Since chromosome bridges,
which represent persistent telophases, cannot occur with-
out mitosis or cell division the effect of high doses is
very likely due to an inhibitory action on the mitotic
process. Following high doses of radiation, this may in
part be a manifestation of damage to the mitotic spindle.
Lack of effect of 250R on overall cell multiplication
probably accounts for the high incidence of chromosome
bridges following this dose of x-rays.

3. Evaluation of the Effect of Various IUDR Concentrations
   on Calf Thyroid Cell Growth

Although IUDR is known to be one of the most
effective analogues for radiosensitization (compared to
5-Bromo-2'-deoxyuridine (BUDR], and 5-Chloro-2'-deoxyuri-
dine [CUDR]), it has been found to be the most toxic to
mammalian cells (Erikson and Szybalski, 1961). This
toxicity is supported by the present study. With this
inherent disadvantage of IUDR in mind, it was decided to
use a primary diploid thyroid cell culture in this study.
The thyroid is known to concentrate one-tenth to one-
fifth of the body iodine in the form of iodonated compounds.
(i.e., mono-iodotryosine, di-iodotyrosine, and thyroxine).
Consequently, by using thyroid cells, at least the cyto-
plasmic toxicity of IUDR would be minimized as it is
evident from growth studies carried out in the presence of
a 4 µg/ml supplement of IUDR (see Figs. 7, 8, and 9).

4. Cytological Examination of X-irradiation Damage to
   Thyroid Cells Cultured in IUDR Supplemented Medium

The incorporation of IUDR into thyroid cells has
been shown in the present investigation, to enhance the
formation of radiation-induced chromosomal bridges. This
radiosensitizing effect of IUDR is in general agreement
with a previous study on survival with other tissues, par-
ticularly human cell lines (Erikson and Szybalski, 1961).
However, the radiosensitization factor observed in these
experiments was approximately 1.3, whilst those of Erikson
and Szybalski (1961) showed at least a two-fold increase
in radiosensitization on the basis of survival studies.
A number of possible alternatives exist which may explain
this discrepancy.

(a) The two cell systems compared are
physiologically different.

(b) Complete scission (by means of ionizing radiation) of a highly polytene chromosome would be expected to be less frequent than scission of a chromosome composed of a smaller number of DNA strands. The thyroid cell chromosome is polytene in nature (Trosko and Wolff, 1963; Heddle and Trosko, 1966). The discrepancy in the degree of radiosensitization between thyroid cells and human cell lines may reflect the difference in the degree of polytenization of their individual chromosomes.

(c) The discrepancy in radiosensitization is due to the difference in chromosome number of the cell lines employed. This explanation is attractive because the parameter for measuring x-irradiation damage is dependent upon chromosomal breakage followed by abnormal restitution giving rise to chromosomal bridges during subsequent mitosis. The cytological expression (CB formation) of IUDR-enhanced x-irradiation damage is self-limiting and its maximum is dependent on target size.

(d) The x-irradiation induced chromosomal breakage, and formation of chromosomal aberrations are not the sole sites involved in cell lethality. It is known that the stages of cell generation (after Howard and Pelc, 1953) at which maximum reproductive damage and maximum chromosomal damage occur do not appear to coincide (Augustein, Mason and Zelle, 1966).

The answer probably lies in a combination of (a), (c) and (d), but results by no means warrant exclusion of the other alternative. Worthy of mention are the findings of Hendrickson (1967) (working with mice in an *in vivo* system) who found that the radiosensitization factor of IUDR amounted to 33% to 35%. This enhancement of radio- sensitivity correlates well with our own findings based on *in vitro* cytological studies.

5. Evaluation of the Effect of Various IUDR Concentrations on Calf Kidney Cell Growth

The results obtained show (see Figs. 17 and 18) that kidney cell growth is depressed by IUDR concentrations as low as 4 µg/ml. There was approximately 25% growth depression by the combined action of 250R x-irradiation and 4 µg/ml of IUDR. This depression was more pronounced at concentrations between 8 and 64 µg/ml; however, the level of toxicity appears to reach threshold saturation, since no further depression was evident when concentrations between 8 and 64 µg/ml were used. This indicates that the cells

(a) incorporate a limited amount of IUDR with the remainder lying exogenous to the cells, and,

(b) must actively metabolize IUDR before growth is affected.

The uptake studies with thyroid cells showed that IUDR was indeed metabolized. A comparison of IUDR treated thyroid and kidney cells indicates that the former is more tolerant to the adverse effects of IUDR at the lower concen- trations, i.e., 4 µg/ml (see Figs. 7, 8, 9, 17 and 18).

6. Cytological Evaluation of X-irradiation Damage of Kidney Cells Cultured in Standard and IUDR Supplemented Media

IUDR was also observed to enhance the radiation- induced formation of chromosome bridges in cultures of kidney

cells (see Figs. 19, 20A and 20B). Sensitization was only increased by a factor of 1.1 as opposed to about 1.3 for thyroid cells. Thus, apparent differences may be explained on the basis of growth depression. In other words, "inhibition of mitosis" precluded cytological detection of some of the x-irradiation damage. When this growth depression factor is considered, then the frequency of CB approaches that found for thyroid cells.

Secondly, the mitotic turnover for kidney cells appears to be higher than that found for thyroid cells (compare Figs. 7 and 17), and undoubtedly influences the results, in that a higher ratio of undamaged cells to damaged cells is apparent and after a shorter period of time.

7. Cytological Evaluation of X-irradiation Damage to
    Thyroid Cells Cultured in ICDR Supplemented Medium

Erickson and Szybalski (1963) demonstrated that ICDR incorporation by the human cell line D98/AG, was similar to that found for IUDR. Furthermore, the degree of x-irradiation sensitization was similar. Their studies suggested agreement with the view that ICDR is converted to IUDR by deamination before incorporation. Since extensive IUDR degradation in vivo is known to occur, and ICDR has been shown to be resistant to this catabolism (Kriss, Tung and Bond, 1962), a comparison of ICDR on growth and radiosensitization was carried out. The results (see Fig. 23) indicated that

(a) ICDR and IUDR are equally effective in
     increasing CB formation at the same
     concentration;

(b) although ICDR is less toxic, concentra-
     tions above that permissible with IUDR
     do not increase effect;

(c) however, since ICDR is not catabolized
     as extensively as IUDR, its use in vivo
     is to be preferred.

8.   Cytological Evaluation of X-irradiation Damage to
     Thyroid Cells Cultured in a Combined IUDR and ICDR
     Supplemented Medium

        It appears that a combination of IUDR and ICDR
does not enhance x-irradiation damage beyond the level
each would achieve, if used independently. This finding
constitutes independent circumstantial evidence for the
view of Welch (1961), Erikson and Szybalski (1963) and
others, that ICDR is deaminated to IUDR prior to incor-
poration. If the two analogues were acting as uniquely
incorporated entities, then some evidence of this should
have appeared in terms of CB frequency. Further evi-
dence for the incorporation of ICDR in the form of IUDR
comes from chromatographic work (see Fig. 12). Chromato-
grams of DNA hydrolysates of cells pre-treated simultan-
eously with IUDR as well as ICDR yielded no "fingerprint"
for ICDR indicating that ICDR is converted into IUDR
prior to DNA incorporation. No apparent advantage is
afforded by the combined use of the two analogues in
conjunction with x-irradiation.

9.   Cytological Evaluation of Continuous 131I-irradiation
     Damage on Thyroid Cells Cultured in Standard and IUDR
     Supplemented Media

        Moore and Colvin (1966) reported the induction
of thyroid cell chromosomal aberrations by 131I-irradiation
in Chinese hamsters, (in vivo), and recorded a high inci-
dence of dicentrics and rings. The results in this thesis
(see Figs. 29, 30, 30A and 30B), show that continuous 131I-
irradiation of thyroid cells in vitro over a four day
period also induced CB.

More specifically, the 131I damage found for IUDR pre-treated cells is similar to that of control cells irradiated with an acute dose of x-rays as well as to that of control cells irradiated with a chronic dose of 131I. This latter information indicates that at the dose level employed (250R) there is no difference in the effectiveness of repair between a dose of acute x-rays and a chronic dose of 131I of same magnitude. 131I and x-irradiation induce the same proportion of chromosome bridges but unlike that with x-rays, IUDR does not sensitize the cells to 131I-irradiation. These alternative responses might be explained by the difference in the 131I beta and x-ray energies. This explanation, however, would appear unlikely, because Erickson and Szybalski (1963) varied x-ray energy over a 20 fold range, and found no difference in radiosensitization of BUDR-grown cells. An alternative explanation might be that the nature of interaction of the two types of irradiation with IUDR-containing DNA is different, as has been found by Delihas, Rich and Eidinoff (1962) for x-rays and neutrons.

10. Effect of Various Treatments on Clone Forming Ability

The experimental conditions under which the cytological studies were conducted could not be completely simulated for clone-forming studies. Although any direct comparison should be made with some reservation, the degree of "mitotic inhibition" (reproductive death) nonetheless was found to be in relatively good agreement with the observed cytological damage caused by x-irradiation.

The degree of "reproductive death" resulting from the use of continuous 131I-irradiation of non-treated and IUDR pre-treated cells was somewhat higher than would be predicted on the basis of cytological evaluation.

The finding that the absence of IUDR radiosensitization of thyroid cells following 131I-irradiation is in

agreement with cytological findings.

  The general agreement between CB and clone-forming ability suggests the existence of a relationship between CB and 'reproductive death" which would bear further investigation.

SUMMARY

1. From cytological evaluation it was concluded that CB
frequency is a reliable parameter of x-irradiation damage
in in vitro thyroid cell systems at low dose levels. How-
ever, when high dose levels were utilized the process of
cell division was impeded, with the result that x-ray
induced damage could not be detected. "Giant cells" as
well as multinucleated cells are thought to be one expres-
sion of this. Thus, a maximum dose level condition (at
which cell division is not retarded for two or three genera-
tions) had to be established if this cytological system
was to be useful in evaluation of x-irradiation damage.
The maximum dose level which meets this condition in the
thyroid cell system was found to be 250R. Growth experi-
ments indicated that this dose did not impede overall
cellular division.

2. Growth evaluation of cells cultured in IUDR supple-
mented medium showed that studies at 4 µg/ml concentrations
could be carried out on thyroid cells without toxicity
becoming the limiting factor. Whether or not this is true
for periods of time beyond the eight to nine days used in
this study is unknown. Extended exposure to IUDR might
show that thyroid cells eventually succumb to IUDR toxicity.

3. From spectrometric, radioautographic and chromatographic
studies, it is concluded that IUDR was incorporated into
the DNA of calf thyroid cells in vitro.

4. Cytological evaluation of IUDR pre-treated thyroid cells
which were x-irradiated, showed an enhanced CB formation
frequency when compared to cells which were x-irradiated
without pre-treatment.

5. A comparison of thyroid and kidney cell systems (By
means of growth and cytological evaluation) showed that
IUDR is more toxic to kidney cells than thyroid cells.

In addition, the response of IUDR pre-treated kidney cells to x-irradiation was smaller than that for thyroid cells. Reasons for this are discussed.

6. Growth evaluation showed ICDR to be less toxic to thyroid cells that IUDR. However, x-irradiation damage was potentiated by ICDR and IUDR to a similar degree.

7. The combined use of IUDR and ICDR showed the maximum radio-potentiation value of the mixture was no higher than if either compound was used independently. It therefore is concluded that the two moieties are incorporated as a single entity and thus, no obvious advantage can be gained by the use of such a combination.

8. A comparison of continuous 131I and x-irradiation showed that the two are about equally effective at producing cell damage in non-treated thyroid cells. However, a comparison of cytological damage induced by the two forms of irradiation on IUDR pre-treated cells showed, that in terms of CB, x-irradiation was more effectively enhanced. It is therefore concluded that 131I-irradiation is not potentiated by IUDR. Possible reasons for this are discussed.

9. Using information derived from an independent experiment (employing clone-forming ability as the parameter of radiation damage) it was concluded that inhibition of mitosis ("reproductive death") does occur, and can be related to CB formation. 131I-irradiation damage is not potentiated by IUDR pre-treatment. CB formation and clone-forming ability could not be established. Although any direct comparison between CB formation and clone-forming ability in control, irradiated (x-ray or 131I) and x-irradiated IUDR or ICDR pre-treated cultures should be made with some reservation, the degree of mitotic inhibition was found to be in relatively good agreement with the observed cytological damage (CB formation) caused by the various treatments.

REFERENCES

Assem, E.S.K. 1965. Metabolic activity of monolayer tissue cultures of human thyroid. Nature 207: 191-192

Atwood, K.C. 1963. In S. Wolff, ed. Radiation - Induced Chromosome Aberrations. Columbia University Press, London and New York, p. 73.

Bain, B. and Lowenstein, L. 1965. In P. S. Russel, H.J. Winn and D.B. Amox, ed., Conference and Workshop on histocompatibility testing. Publication 1229, National Academy of Sciences - National Research Council, Washington, D. C., p. 199.

Bergonie, J. and Tribondeau, L. 1906. Interpretation de quelques resultants de la radiotherapie et essai de fixation d'une technique rationelle. Compt. Rend. Acad. Sci. 143: 983-985.

Chang, P. and Welch, A.D. 1961. Preparation of 5-iodo-2'-deoxycytidine. Biochem. Pharmacol. 8: 327-328.

Cheong, L., Rich, M.A. and Eidinoff, M.L. 1960. Introduction of the 5-halogenated uracil moiety into deoxyribonucleic acid of mammalian cells in cultures. J. Biol. Chem. 235: 1441-1447.

Chu, E.H.Y. 1965a. In S.J. Geerts, ed., Proc. 11th Intern. Congr. Genet. The Hague. Permagon Press, Oxford. p. 85.

Cramer, J.W., Prusoff, W.H. and Welch, A.D. 1961. 5-bromo-2'-deocycytidine (BCDR) II. Studies with murine neoplastic cells in culture and in vitro. Biochem. Pharmacol. 8: 331-335.

Cramer, J.W., Prusoff, W.H., Welch, A.D. Sartorelli, A.C., Delmore, J.W., von Essen, C.F. and Chang, P.K., 1962. Studies on the biochemical pharmacology of 5-iodo-2'-deoxycytidine in vitro and in vivo. Biochem. Pharmacol. 11: 761-768.

Davies, O.R. and Evans, H.J. 1966. In L.G. Augustein,
    O.R. Mason and M.R. Zelle, ed., Advances in Radiation
    Biology. Academic Press, London. p. 283.

Delihas, N., Rich, M.A. and Eidinoff, M.L. 1962. Radio-
    sensitization of a mammalian cell line with 5-bromo-
    deocyuridine. Rad. Res. 17: 479-491.

Dewey, W.C. and Humphrey, R.M. 1965. Increase in radio-
    sensitivity to ionizing radiation treated to replace-
    ment of thymidine in mammalian cells with 5-bromo-
    deoxyuridine. Rad. Res. 26: 538-553.

Djordjevic, B. and Szybalski, W. 1960. Genetics of human
    cell lines. III. Incorporation of 5-bromo and 5-iodo-
    deoxyuridine into the deoxyribonucleic acid of human
    cells and its effect on radiation sensitivity. J. Exp.
    Med. 112: 509-531.

Eidinoff, M.L., Cheong, L. and Rich, M.A. 1959. Incorpora-
    tion of unnatural pyrimidine bases into deoxyribonuc-
    leic acid of mammalian cells. Science 129: 1550-1551.

Erikson, R.L. and Szybalski, W. 1961. Molecular radiobiology
    of human cell lines. I. Comparative sensitivity of
    x-rays and ultra-violet light of cells containing
    halogen-substituted DNA. Biochem. Biophys. Res. Commun.
    4: 258-261.

Erikson, R.L. and Szybalski, W. 1963. Molecular radiobiology
    of human cell lines V. Comparative radiosensitizing
    properties of 5-halodeoxycytidines and 5-halodeoxyuri-
    dines. Rad. Res. 20: 252-262.

Frisch, P.M. and Visser, P.W. 1959. 5-bromo-deoxycytidines
    and 5-chloro-deoxycytidine. J. Am. Chem. Soc. 81: 1756-
    1758.

Greer, S. and Zamenhof, 1957. Effect of 5-bromouracil in
    deoxyribonucleic acid of E. coli on sensitivity to ultra-
    violet irradiation. Abst. 131 meeting Am.Chem.Soc. p.3c.

Greer, S. 1960. Studies on ultra-violet irradiation of E. coli containing 5-bromouracil in its DNA. J. Gen. Micro. 22: 618-634.

Heddle, J.A. and Trosko, J.E. 1966. Is the transition from chromosome to chromatid aberrations the result of the formation of single-stranded DNA? Exp. Cell Res. 42: 171-177.

Hendrickson, F.R. 1967. Intestinal injury in mice using combined radiation and chemotherapy. Abst. 15th Annual Meeting of the Rad. Res. Soc. 31: 601.

Howard, A. and Pelc, S. 1953. Synthesis of deoxyribonucleic acid in normal cells and irradiated cells and its relation to chromosome breakage. Heredity Suppl. 6: 261-273.

Hung, W. and Winship, T. 1964. Hormone synthesis by normal human thyroid cells in tissue cultures. Proc. Soc. Exp. Biol. and Med. 116: 887-890.

Irvine, J.W. 1960a. An investigation of the pathogenesis of Hashimotos' disease by thyroid tissue culture. J. Endocr. 20: 83-90.

Kaplan, H.S., Smith, K.C., and Tomlin, P.A. 1962. Effect of halogenated pyrimidines on radiosensitivity of E.coli. Rad. Res. 16: 98-113.

Kaul, B.L. and Natarajan, A.T. 1966. Radiomimetric and radio-sensitizing effects of 5-bromouracil and 2-amino purine in root meristems of barley and onion. Ind. J. Exp. Biol. 4: 109-113.

Kihlman, B.A. 1963. The effect of 5-halogenated deoxyuridines on the frequency of x-ray induced chromosomal aberrations in Vicia faba. Hereditas 49: 353-370.

Koller, P.C. 1947. The effect of radiation on the normal and malignant cell in man. Brit. J. Radiol. Suppl. 1: 84-98.

Koo, F.K.S. 1963. Synergistic effect of 5-bromodeoxyuridine and gamma rays on chromosomes. Science 141: 261-262.

Kriss, J.P., Tung, L. and Bond. S. 1962. The distribution and fate of iododeoxycytidine in the mouse and rat. Cancer Res. 22: 1257-1264.

Litman, R.M. and Pardee, A.B. 1960. The induction of mutants of bacteriophage T_2 by 5-bromouracil. III. Nutritional and structural evidence regarding antagenic action. Biochem. Biophys. Acta. 42: 117-130.

Littlefield, J.W. and Gould, E.A. 1960. The toxic effect of 5-bromodeoxyuridine on cultures epithelial cells. J. Biol. Chem. 235: 1129-1133.

Loeninger, R., Holt, J.G. and Hine, G.J. 1956. In G.J. Hine and G.L. Brownell, ed., Radiation Dosimetry. Academic Press Inc., New York. p. 826.

Mallette, J.M. and Anthony, A. 1966. Growth in culture of trypsin dissociated thyroid cells from adult rats. Exp. Cell Res. 41: 642-651.

Marcus, P.J., Cieciura, S.J. and Puck, T.T. 1956. Clonal growth in vitro of epithelial cells from normal human tissues. F. Exp. Med. 104: 615-628

Merchant, D.J., Kahn, R.H. and Murphy, W.H., Jr. 1960. In Handbook of Cell and Organ Culture. Burgess Publishing Company, Minneapolis. p. 162, 177.

Moore, W., Jr. and Colvin, M. 1966. The effect of 131I on the aberration rate of chromosomes from Chinese hamster thyroids. Int. J. Rad. Biol. 10: 391-401.

Muller, H.J. 1927. Artificial transmutation of the gene. Science 66: 84-87.

Opara-Kubinska, J., Lorkiewicz, S. and Szybalski, W. 1961. Genetics transformation studies. II. Radiation sensitivity of halogen labelled DNA. Biochem. Biophys. Res. Commun. 4: 288-291.

Parker, R.C. 1961. In Methods of Tissue Culture. Hoeber, New York. p. 199.

Puck, T.T. and Marcus, P.I. 1955. A rapid method for viable cell titration and clone production with HeLa cells in tissue culture: The use of x-irradiated cells to supply conditioning factors. P.N.A.S. 41: 432-437

Puck, T.T., Marcus, P.I. and Cieciura, S.J. 1956. Clonal growth of mammalian cells in vitro. Growth character- istics of clones from single HeLa cells with and without a "feeder" layer. J. Exp. Med. 103: 273-284.

Puck, T.T. and Marcus, P.I. 1956. Action of x-rays on mam- malian cells. J. Exp. Med. 103: 653-666.

Puck, T.T. 1960. Radiation and the human cell. Scient. Amer. 202. (#4): 142-153.

Pulvertaft, R.J.V., Davis, J.R., Weiss, L. and Wilkinson, J.H. 1959, Studies on tissue cultures of human pathological thyroids. J. Path. Bact. 77: 19-32.

Ragni, G. and Szybalski, W. 1962. Molecular biology of human cell lines. II. Effects of thymidine replacement by halogenated analogues on cell inactivation by decay of incorporated radio phosphorous. J. Mol. Biol. 4: 338-346.

Stadler, L.J. 1928. Genetic effects of x-rays in maize. P.N.A.S. 14: 69.

Tong, W., Kerkoff, P. and Chaikoff, I.L. 1962. Iodine metab- olism of dispersed thyroid cells obtained by trypsiniza- tion of sheep thyroid glands. Biochem. Biophys. Acta. 60: 1-19.

Trosko, J.E. and Wolff, S. 1963. Strandedness of Vicia faba chromosomes as revealed by enzyme digestion. J. Cell Biol. 26: 125-135.

- 135 -

Welch, A.D. 1961. Some metabolic approaches to cancer chemotherapy. Cancer Res. 21: 1475-1490.

Whitfield, J.F., Rixon, R.H. and Rhynas, P.O.W. 1959. Cytological consequences of x-irradiation in mouse L-cells. Exp. Cell Res. 18: 591-593.

Yu, C.K. 1966. Polycentric chromosomes induced by x-rays in giant cells of Chinese hamster cells in vitro. Chromosoma 18: 60-69.

Zeigler, C.A., Chleck, D.J. and Brinkerhoff, J. 1957. In G.G. Bell, Jr., and R.N. Hayes, ed., Liquid scintillation counting. Pergamon Press, New York. p. 186.